DOCTORAL THESIS

Contribution on Cellulose Nano-Biotechnology for Food Bioprocessing

Mrinal Nishant Kumar

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Mrinal Nishant Kumar

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Επταμελής Εξεταστική Επιτροπή

Τριμελής Συμβουλευτική Επιτροπή

Αργυρώ Μπεκατώρου, Επίκουρος Καθηγήτρια (Επιβλέπουσα)
Τμήμα Χημείας, Πανεπιστήμιο Πατρών

Αθανάσιος Α. Κουτίνας, Ομότιμος Καθηγητής
Τμήμα Χημείας, Πανεπιστήμιο Πατρών

Μαρία Κανελλάκη, Καθηγήτρια
Τμήμα Χημείας, Πανεπιστήμιο Πατρών

Εξωτερικοί εξεταστές

Ashok Pandey, Καθηγητής
Biotechnology Division, National Institute for Interdisciplinary Science & Technology, CSIR, Trivandrum, India

Εμμανουήλ Παπαμιχαήλ, Καθηγητής
Τμήμα Χημείας, Πανεπιστήμιο Ιωαννίνων

Μαγδαληνή Σουπιώνη, Επίκουρος Καθηγήτρια
Τμήμα Χημείας, Πανεπιστήμιο Πατρών

Σταύρος Πλέσσας, Επίκουρος Καθηγητής
Τμήμα Αγροτικής Ανάπτυξης, Δημοκρίτειο Πανεπιστήμιο Θράκης
Seven Member Examination committee

Three Member Advisory Committee

Argyro Bekatorou, Assistant Professor (Supervisor)
Department of Chemistry, University of Patras, Greece

Athanasios A. Koutinas, Professor Emeritus
Department of Chemistry, University of Patras, Greece

Maria Kanellaki, Professor
Department of Chemistry, University of Patras, Greece

External Examiners

Ashok Pandey, Professor
Biotechnology Division, National Institute for Interdisciplinary Science & Technology, CSIR, Trivandrum, India

Emmanuel M. Papamichael, Professor
Department of Chemistry, University of Ioannina, Greece

Magdalini Soupioni, Assistant Professor
Department of Chemistry, University of Patras, Greece

Stavros Plessas, Assistant Professor
Department of Agricultural Development, Democritus University of Thrace, Greece
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“The method of scientific investigation is nothing but the expression of a necessary mode of working of the human mind”

Thomas Henry Huxley
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Abbreviations

BC: Bacterial cellulose
CA: Cellulose acetate
CAB: Cellulose acetate butyrate
CAP: Cellulose acetate propionate
FTIR: Fourier transform infrared spectroscopy
LAF: Lactic acid fermentation
P(3HB-co-3HV) (or PHBV): Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P3HB (or PHB): Poly(3-hydroxybutyrate)
PBSu: Poly(butylene succinate)
PCL: Polycaprolactone
PESu: Poly(ethylene succinate)
PGA: Polyglycolate
PHAs: Polyhydroxyalkanoates
PHB: polyhydroxybutyrate
PHBs: Polyhydroxybutyrates
PLA: Polylactic acid
PPAd: Poly(propylene adipate)
RWC: Relevant water content
SPME: Solid phase microextraction
TC: Tubular cellulose (delignified cellulosic material with tubular porous structure)
XRD: X-ray diffractometry
**Abstract**

The need for innovative and sustainable technologies in the area of food bioprocessing and fermentation technology has brought upon a great interest in the use of cheap renewable resources in the form of raw materials available in their natural existence. Wood sawdust and rice husk are such ubiquitous lignocellulosic biomasses that can be used as novel biodegradable micro/nano-tubular cellulosic materials (TCs) after a delignification process, alone or as composites with natural microbial biopolymers such as polylactic acid (PLA) and polyhydroxybutyrate (PHB), to design natural microbial biocatalysts for food bioprocessing.

Specifically, TC from wood sawdust of *Mangifera indica* (mango tree) and *Shorea robusta* (sal tree), and husks of *Oryza sativa* (rice) of Indian origin were delignified and characterized in this study, and were used in various fermentation processes. Lignin was removed from the cellulose matrix by alkaline treatment to leave behind a porous, tubular structure. Surface characteristics, lignin content and other chemical components were analyzed to determine the chemical and structural differences among the cellulosic materials before and after the delignification treatment.

The proximate analysis from the leaves, sawdust and husk extracts of the plant materials included the estimation of total carbohydrates, reducing sugars, nitrogen, crude protein, ash, relative water content (RWC). The results showed higher percentage of nitrogen, crude protein, ash and RWC in the leaves of sal than its sawdust. On the other hand, lower percentage of only RWC was found in the leaves of mango and rice compared to their sawdust and husk, respectively. This study will further help to set-up certain protocols for purification of the extracts as porous biopolymer materials for various applications.

In the next part, carbonization of the TCs under nitrogen flow was done using various temperature/time combinations in order to increase their specific surface area. The materials were studied by TGA/DTA, SEM and BET analysis. Atomic Force Microscopy, X-Ray Diffractometry (XRD) and FTIR analyses where also carried out in order to study the qualitative differences between the TCs and carbonized TCs.

The presence of tubular structure in the delignified materials at the scale of 60-100 µm was observed distinctively through SEM analyses, whereas for the carbonized TCs a bristle and more crystalline form of structures were observed at the scale of 20-100 µm. The TGA/DTA analyses showed the mass change behavior of Indian origin TCs with progressive weight loss at a temperature of 350°C. The surface area and pore diameters were found to be of 3-4 fold increase in the carbonized TCs compared to the delignified TCs. The results from XRD showed the variation in the degree of crystallinity (CI) for delignified and carbonized TCs. The FT-IR analyses
were done to monitor the chemical structure by identifying the functional groups present in each sample after delignification and carbonization, confirming loss of the most amount of lignin during the delignification process and decrease in the intensity of bands during carbonization.

These cellulosic materials were evaluated as biofilters for cold pasteurization of drinkable water and for skimmed and semi-skimmed milk at 4°C, with a flow rate of 2 L/day using a one-way peristaltic pump. Regeneration of the filters was done using hot water. The microorganisms used for deliberate contamination of drinkable water were Lactobacillus bulgaricus and Saccharomyces cerevisiae AXAZ-1 and only L. bulgaricus for milk. The nano/micro-porous TC filters where packed in a nylon thin perforated fabric placed in a bioreactor with stainless steel covers. Both inflow and outflow were analyzed using the standard plate count technique and optical density (OD) determinations at 600 and 700 nm. The efficiency of the mango and sal TC was higher than the rice husk TC in all cases. The microbial removal load ranged from 80-100 % for S. cerevisiae and 70-90 % for L. bulgaricus.

Furthermore, novel biodegradable composite materials based on the mango, sal and rice TCs and microbial polymers (PLA and PHB), were prepared and characterized. The microbial polymers were encapsulated separately in sodium alginate beads forming a (PLA-alginate) matrix. TC was selected because it exhibits high purity, high mechanical strength and an ultra-fine fibrous 2D network structure, while PLA is a low cost, biodegradable matrix derived from various natural resources. PHD on the other hand is more expensive and less stable material at high temperatures - it has low mechanical strength and undergoes thermal degradation-and therefore was not further investigated.

The plain TCs and their composites were evaluated as cell immobilization carriers for use as biocatalysts in lactic acid and alcoholic fermentations. In both cases of fermentations (lactic and alcoholic) and types of biocatalysts (cells immobilized on plain or composite materials), improvements were observed compared to free cells. Lower fermentation rates, higher product yields and productivities and higher amounts of flavor-active volatile by-products were achieved. The product was assayed by various analytical techniques including GCMS, HPLC, and GC. Thus the use of composite biocatalysts for co-immobilization of different microorganisms or enzymes (in separate layers of the biocatalysts), to efficiently conduct different types of fermentations in the same bioreactor is proposed. Such biocatalysts may help avoid inhibition problems of chemical or biological nature (e.g. species competition).

**Keywords:** Food bioprocess development, Biomass utilization, Cold pasteurization, Nano/micro porous cellulose, Immobilized cells, Lactic acid fermentation, Alcoholic fermentation
Περίληψη

Η ανάγκη για καινοτόμες τεχνολογίες στον τομέα της επεξεργασίας τροφίμων και των ζυμώσεων έχει επικεντρώσει το ενδιαφέρον στη χρήση ανανεώσιμων μη επεξεργασμένων πρώτων υλών χαμηλού κόστους.

Ο φλοιός ρυζιού και το πριονίδι ξύλου αποτελούν λιγνοκυτταρινούχα υλικά που μπορούν να χρησιμοποιηθούν ως καινοτόμα βιοδιασπώμενα μικρο/νανο-πορώδη κυτταρινούχα υλικά (ΠΚΥ). Μετά από απολιγνινοποίηση, τα ΠΚΥ μπορούν να χρησιμοποιηθούν είτε αυτούσια ή σε συνδυασμό με μικροβιακά βιοπολυμερή όπως το πολυγαλακτικό (PLA) και το πολυ-υδροξυ-βουτυρικό οξύ (PHB), για το σχεδιασμό βιοκαταλυτών στα πλαίσια βιοδιεργασιών τροφίμων. Συγκεκριμένα, στην παρούσα εργασία χρησιμοποιήθηκαν ΠΚΥ από πριονίδι ξύλου των δέντρων Mangifera indica (δέντρο μάνγκο), Shorea robusta (δέντρο Σάλ), και των φλοιών του Oryza sativa (ρύζι) ινδικής προέλευσης. Έγινε απομάκρυνση της λιγνίνης από το κυτταρινούχο υλικό με τη χρήση αλκαλικού διαλύματος. Προσδιορίστηκαν τα χαρακτηριστικά της επιφάνειας, η περιεκτικότητα της λιγνίνης και άλλων χημικών συστατικών με στόχο την ανάδειξη των δομικών και χημικών διαφορών των κυτταρινούχων υλικών πριν και μετά την απολιγνινοποίηση. Η χημική ανάλυση των φύλλων, πριονιδίου και εκχυλίσματος των φλοιών των μάνγκο, σάλ, και ρυζιού περιλαμβάνει τον προσδιορισμό των συνολικών υδατανθράκων, αναγόντων σακχάρων, αζώτου, πρωτεΐνης, τέφρας, και υγρασίας. Τα αποτελέσματα έδειξαν ότι τα φύλλα του φυτού σάλ, περιείχαν μεγαλύτερη μεγελότερη συγκέντρωση αζώτου, πρωτεΐνης, τέφρας και νερού συγκριτικά με το πριονίδι. Ωστόσο, στην περίπτωση του δέντρου μάνγκο και του ρυζιού, η περιεχόμενη υγρασία ήταν μικρότερη στα φύλλα σε σύγκριση με το πριονίδι και το φλοιό. Η υγρασία του πριονιδίου του μάνγκο και του ρυζιού ήταν υψηλότερη από το σάλ, του οποίου τα φύλλα περιείχαν μεγαλύτερο ποσοστό υγρασίας. Η παρουσία μελέτη θα βοηθήσει στην οργάνωση πρωτοκόλλων για την εκχύλιση φυτικών βιοπολυμερών με μεγάλο πορώδες για εφαρμογές στη νανο/μικρο-βιοτεχνολογία.

Στο δεύτερο μέρος, μελετήθηκε η εξανθράκωση των κυτταρινούχων υλικών υπό ροή αζώτου σε διάφορες θερμοκρασίες και χρονική διάρκεια με στόχο την αύξηση της ειδικής τους επιφάνειας. Η μελέτη των υλικών έγινε με αναλύσεις προσρόφησης-εκρόφησης αζώτου (BET), θερμοσταθμική ανάλυση (TGA/DTA) και παρατήρηση σε ηλεκτρονικό μικροσκόπιο σάρωσης (SEM). Παράλληλα, πραγματοποιήθηκαν αναλύσεις φασματοσκοπίας υπερύθρου-μετασχηματισμού Fourier (FT-IR) και περίθλασης ακτίνων-Χ (XRD) για την ποιοτική ανάλυση των απολιγνινοποιημένων και εξανθρακωμένων ΠΚΥ. Η παρατήρηση στο ηλεκτρονικό μικροσκόπιο έδειξε την παρουσία σωληνωτής δομής με διάμετρο της τάξης 60-100 µm και 20-100 µm για τα
ανεπεξέργαστα και εξανθρακωμένα υλικα αντίστοιχα. Η θερμοσταθμική ανάλυση έδειξε ότι η μεγαλύτερη μεταβολή της μάζας των ΠΚΥ ινδικής προέλευσης έγινε στους 350°C. Η ειδική επιφάνεια και η διάμετρος των πόρων των εξανθρακωμένων υλικών ήταν 3-4 φορές μεγαλύτερες απ' ότι στα ΠΚΥ. Ο βαθμός κρυσταλλικότητας παρουσίασε διακυμάνσεις μεταξύ των ανεπεξέργαστων και των εξανθρακωμένων ΠΚΥ. Η φασματοσκοπία υπερύθρου έγινε για την ανάλυση της μεταβολής της χημικής δομής των υλικών μετά την απολιγνινοποίηση και την εξανθράκωση, μέσω της ταυτοποίησης των δομικών ομάδων. Τα φάσματα FT-IR παρουσίασαν πληροφορίες για την δομή και τα μοριακά χαρακτηριστικά των υλικών επιβεβαιώνοντας την απομάκρυνση της λιγνίνης κατά την απολιγνινοποίηση και τις δομικές αλλαγές κατά την εξανθράκωση.

Στη συνέχεια, αξιολογήθηκε η χρήση των ΣΚ ως βιοφίλτρα για την ψυχρή παστερίωση πόσιμου νερού (μικροβιακό φορτίο 0.2, 0.5 g/L, μήκος φίλτρου 35 και 45 cm) και αποβουτυρωμένου, ημι-αποβουτυρωμένου γάλακτος (μικροβιακό φορτίο 0.1, 0.2 g/L, μήκος φίλτρου 35 και 45 cm) στους 40°C με ροή 2 L/ημέρα χρησιμοποιώντας περισταλτική αντλία. Η αναγέννηση των φίλτρων έγινε με χρήση ζεστού νερού. Οι μικροοργανισμοί που χρησιμοποιήθηκαν για την επιμόλυνση πόσιμου νερού ήταν ο Lactobacillus bulgaricus και ο Saccharomyces cerevisiae AXAZ-1, ενώ για το γάλα μόνο ο L. bulgaricus. Τα ΠΚΥ φίλτρα τοποθετήθηκαν σε νάιλον διάτρητο ύφασμα και στη συνέχεια στον βιοαντιδραστήρα με ανοξείδωτα καλύμματα. Στα δείγματα της εισόδου και της εξόδου έγιναν μικροβιολογικές αναλύσεις καθώς και μετρήσεις της οπτικής πυκνότητας στα 600 και 700 nm. Η αποτελεσματικότητα των ΠΚΥ μάνγκο και σάλ ήταν υψηλότερη του φλοιού ρυζιού σε όλες τις περιπτώσεις. Η μικροβιακή απομάκρυνση κυμάνθηκε μεταξύ 80-100% για τον μικροοργανισμό S. cerevisiae και 70-90% για το βακτήριο L. bulgaricus.

Παράλληλα, παρασκευάστηκαν και χαρακτηρίστηκαν καινοτόμα βιοδιασπώμενα σύνθετα υλικά αποτελούμενα από ΠΚΥ ινδικής προέλευσης και μικροβιακά πολυμερή (PLA, PHB). Τα μικροβιακά πολυμερή ενθυλακώθηκαν ξεχωριστά σε σφαιρίδια αλγινικού νατρίου. Τα ΠΚΥ και τα σύνθετα υλικά αξιολογήθηκαν ως φορείς ακινητοποίησης κυττάρων L. bulgaricus και S. cerevisiae για γαλακτική και αλκοολική ζύμωση, αντίστοιχα. Και στις δυο περιπτώσεις πραγματοποιήθηκαν επαναλαμβανόμενες παρτίδες ζύμωσης και τα ακινητοποιημένα κύτταρα αποδείχθηκαν πιο αποτελεσματικά σε σχέση με τα ελεύθερα, ακόμη και σε χαμηλές θερμοκρασίες. Και στις δυο περιπτώσεις πραγματοποιήθηκαν επαναλαμβανόμενες παρτίδες ζύμωσης και τα ακινητοποιημένα κύτταρα αποδείχθηκαν πιο αποτελεσματικά σε σχέση με τα ελεύθερα, ακόμη και σε χαμηλές θερμοκρασίες. Και στις δυο περιπτώσεις πραγματοποιήθηκαν επαναλαμβανόμενες παρτίδες ζύμωσης και τα ακινητοποιημένα κύτταρα αποδείχθηκαν πιο αποτελεσματικά σε σχέση με τα ελεύθερα, ακόμη και σε χαμηλές θερμοκρασίες.
Λέξεις Κλειδιά: Ανάπτυξη βιοδιεργασιών τροφίμων, Χρήση βιοκαταλυτών, Ψυχρή παστερίωση, Νάνο/μίκρο πορώδης κυτταρίνη, Ακινητοποιημένα κύτταρα, Γαλακτική ζύμωση, Αλκοολική ζύμωση
1. Introduction

Chapter 1 - Nanotechnology

1.1 Recent advances and approach

The natural significance of advances and refinements of knowledge on creative manipulation of materials has emphasized on the growing interest in nanostructured materials. Nano-materials have unusual and unpredictable characteristics. The distinctive research contributions to accomplishments characterized by the prefix “nano”, from physical properties to chemistry and to ethical/environmental concerns, are currently areas of great interest (USKOKOVIC, 2007). However, while research contribution in making artificial changes in the features of nature has increased over time routinely producing extraordinary complex nanostructures, it was established that nanoparticles have been present since the evolution of planetary life and the earliest appearance of mankind’s ability to produce arts, tools, and machinery (HOCHELLA et al., 2002; ARANTEGUI et al., 2003).

Today’s fanatical interest in nanomaterials has undoubtedly been supported by large investment funds focusing on the creation of ever-finer technological products. This technology is considered to be a useful tool in providing knowledge on biochemical and physical phenomena at the nanoscale with design of new materials with novel properties and functions for a wide range of applications, of which biosensors currently represent one of the main pervasive results of this discipline (JIANRONG et al., 2004; SCOGNAMIGLIO, 2013).

The word “nanotechnology” was first introduced by a Japanese engineer, Norio Taniguchi (TANIGUCHI, 1974). This implied a new technology that went beyond controlling materials and engineering on the micrometer scale that had conquered the 20th century. However, due to many misconceptions, the latter approach to nanodesign is today generally referred to as “molecular assembly.” Nanoscience is here defined as “the study of phenomena and the manipulation of physical systems that produce significant information (i.e. readable differences) on a spatial scale known as “nano” \((10^{-9} \text{ m} = 1 \text{ nm})\) with critical boundaries that do not exceed 100 nm in length at least in one direction” (BATESON, 2002). Therefore, nanotechnologies focus on the design, characterization, production, and application of nanoscale systems and their components. The boundaries between the physical regions of macroscopic, microscopic, and nanoscopic are not sharp and they depend on the effects being considered (WAUTELET, 2001).
The proposed term nanotechnology has been publicized as the next revolution in many industries, including food processing and packaging. The applications of nanobased technology in food industry may include nanoparticulate delivery systems (e.g. micelles, liposomes, nanoemulsions, biopolymeric nanoparticles and cubosomes), food safety and biosecurity (e.g. nanosensors), and nanotoxicity (CHEN et al., 2006; MAYNARD, 2006). Although many food researchers would claim that the industry has already embraced nanotechnology, only limited research in nanotechnology has been performed in concern to food and food-related products. The global development of nanofoods is indeed on its initial stage. In fact, the food industry is only beginning to realize the full potential of nanotechnology. Around the world, the number of newly developed applications of nanotechnology in food is growing rapidly (SANGUANSRI & AUGUSTIN, 2006).

1.1.1 Objectives and outcomes

The current challenges in nanotechnology or nanobiotechnology have stimulated a broad research community. The complexity of molecular interactions and nanostructures, and the diversity of strategies and applications, clearly require a multidisciplinary scientific approach. A creative translation from the biological to the technological domain with the invention of new applications is necessary. The increase in convolution of diverse materials that involve multiple scales, the combination of rational design and evolution, and the emerging interaction of “bio” and “nano” are some vast technological trends that accelerate the nanotechnology. Thus, this fresh, young and innovative research area has mushroomed up into a responsible and strong multidisciplinary endeavor that will continue to influence both fundamental and applied areas of research (HESS & JAEGER, 2010).

Research on food bioprocess technology development has been extensive during the last decades; however most of these technologies have not been used at industrial level. The main problems associated with these technologies are related to productivity, ease of industrial application and production cost. However there are still opportunities to use abundant, low cost materials with specific chemical or nanomechanical properties to create multiple new, effective bioprocessing systems (KOUTINAS et al., 2012). Nanoscience is one of the emerging fields for new product development, along with the understanding of complex interactions and molecular connection among the food components.

The use of biopolymers in this concern is now an up growing interest to deal with liquid food treatment. For example, delignified cellulosic material has shown a significant promotional effect on the alcoholic fermentation as yeast immobilization support. However, its potential for further biotechnological development is unexploited. Characterization of this tubular cellulosic material (TC) showed that its micro and nanoporous structure justified its promoting activity in bioprocessing (fermentation). Moreover, TC was considered a suitable material for novel applications such as cold pasteurization of liquid foods, which is one of the aims of this thesis, and new perspectives in research such as the development of new composite materials, templates for cylindrical nanoparticles, etc. (KOUTINAS et al., 2012).
Progressive evaluation and advances in the designing of nano/micro-structural materials have made possible accordingly their application in the food and related industries. At various macroscopic levels, the food structure, rheology, sensory properties and their microbial loads are found to be influenced. One of the major problems of food and drug production is the use of the relatively costly pasteurization processes. Thus TC from different wood sawdust in this concern was considered as a most effective source for the design and development of nano/micro-tubular microbial filters. The area of research is currently been emphasized in this review with its further understanding to implement its application for cold pasteurization of liquid foods.

1.1.2 Statement of problems and opportunities for food processing

Food is a fuel for life as the word itself emphasizes on the importance of its presence. Thus there has been a continuous increasing requirement for novel developments on its preservation, processing, spoilage, and storage, with sustainability and cost effective benefits. The increasing demand on food safety and its upgrading though the use of nanotechnological applications, have led to extensive research on the use of nanomaterials for food processing. In this context, efforts are being made on the development of novel polymer matrix composites for a variety of industrial applications. For food applications, these materials should be of food grade purity, have low specific gravity and exhibit good mechanical strength (HULL & CLYNE, 1996; JANG, 1994; JONES, 1994).

Chapter 2 - Food preservation

1.2.1 Food preservation

Technology transfer through knowledge and commercial valorization is increasing more efficiently in the evolution of the modern chemistry. Preservation in this reference is the only way to extend the shelf-life of raw materials or a food product beyond their “natural” (i.e. without human intervention) decay times. This extension of shelf-life is considered to be a competitive interaction between different physicochemical or biochemical processes depending upon the growth and development of different microbial populations that could be sometimes beneficial or more commonly detrimental for maintaining desirable food properties.

Application of Ultrasound technology in food for processing, preservation, and extraction is considered to be one of the important wheels of development in this sector. The applications of ultrasound in food processing mainly targets cooking, freezing/crystallization, drying, pickling/marinating, degassing, filtration, demoulding, emulsification, oxidation, cutting and defoaming. Food products such as fruit and
vegetables, fat and oils, sugar, dairy, meat, coffee and cocoa, meat and flours, are complex mixtures of proteins and lipids, sugars, fibers, vitamins, aromas, pigments, antioxidants, and other organic and mineral compounds. In context to their processing most of the food ingredients and products are thermally sensitive and susceptible to physical, chemical and microbiological changes. Thus deterioration in the quality of natural food compounds due to prolonged heating and cooling led to seeking new sustainable green and innovative techniques in processing, pasteurization and extraction, which typically involve less time, water and energy such as ultrasound assisted processing (CHEMAT et al., 2011).

Methods employed for defoaming have also brought upon a lot of interest. Foam is defined as a dispersion of gas in liquid, with its density approaching equal to that of the gas. Application of defoaming techniques has been widely used for industrial processing for food production, pharmaceuticals, agroindustries, and cosmetics. In food manufacturing, foam has historically been controlled by the use of mechanical breakers, lowering packaging container temperatures below the ambient environment, or by the addition of chemical anti-foams (MASON et al., 2005; MOREY et al., 1999).

However, the major focus in food processing emphasizes on the removal and inactivation of microorganisms and enzymes as a means of preservation by the application of physical energy in the form of heat with high output. Although heat can obviously sustain food preservation, it also causes some deterioration, resulting in the loss of valuable nutrients, vitamins, and reduction of organoleptic properties. This has brought upon the interest of the researchers to design alternative methods for food preservation that involve different strategies and physical principles.

The most important factor in the selection of the preservation technique depends upon the characteristic of the food to be preserved and the microorganisms of concern. The various methods to control and reduce microbial contamination include (1) low temperature storage (freezing-refrigeration), (2) high temperature processing (cooking-pasteurization-sterilization), (3) irradiation, (4) moisture control and water activity reduction (dehydration-humectants), (5) control of pH, (6) chemical additives (natural/synthetic antimicrobials), (7) fermentation and bio-preservation, (8) packaging and changes in gas atmosphere, and (9) interactions of factors or multiple hurdles that can affect the growth of microorganisms depending upon the type of food and their origin. With the change of times there has been much influential modification in the existing technologies for food microbial inactivation (SOFOS, 1993). However, complementary food preservation techniques such as modified atmosphere, addition of preservatives or the use of refrigerated storage and distribution might be needed to control the growth of the surviving microorganisms (FILIPA et al., 2012). Most of the modern food preservation technologies involve thermal processing, chilling and freezing, concentration, drying, ionizing radiation, chemical preservation, high hydrostatic pressure, pulsed electric field, and intense light. Hurdle-effect technology is another kind of preservation method that involves the application of combined preservation techniques.
1.2.2 Food spoilage

The main objective of any food processing is to prevent and control spoilage and maintain food quality last over a certain duration which is known as shelf-life. Food spoilage can be of different types such as microbial, enzymatic, chemical, and physical, of which microbial spoilage is the most important as it affects food quality and safety directly. Microbial spoilage is mainly caused due to the deteriorating activity of the microorganisms present in food. E.g. *Saccharomyces*, *Aspergillus*, and *Penicillium* species are some of the most common form of fungi that cause food spoilage through the secretion of lipase and proteinase enzymes. Enzymatic spoilage can happen due to enzyme catalyzed reactions that cause undesirable changes in the food. Chemical spoilage on the other hand is due to non-enzymatic chemical reactions that occur between the food ingredients (e.g. Maillard browning), or between the food and its environment (e.g. lipid oxidation). Physical spoilage lastly is the result of undesirable changes in the physical structure of the food (e.g. sugar crystallization, emulsion separation, collapse of gels, etc.). Therefore, food spoilage in simple terms is a process that leads to the deterioration of the sensory quality (taste, flavor, texture, color and appearance), safety and nutritional value of the food (BERK, 2013).

It is estimated that about 20% of the food worldwide is wasted due to spoilage caused by moulds, food-borne pathogens and bacteria. Thus with the increasing concern for food safety issues related to pathogenicity as well as adverse health effects (e.g. carcinogenicity) of conventional chemical preservatives such as nitrite, benzoate and sorbate, natural anti-microbial agents have received great attention due to their well-documented safety profiles. For example, monolaurin, a naturally existing substance in plants and in the milks of humans and animals, has been reported to provide energy, reduce blood fat and cholesterol levels, lower blood pressure, and prevent body fat accumulation (TAKEUCHI et al., 2006; THOLSTRUP et al., 2004).

Organisms that cause food spoilage are present everywhere in the air, soil and water. Enzymes that may cause undesirable changes in flavor, color and texture are present in raw materials. Therefore, the major challenge for food safety and quality is to deal with food production and distribution in order to satisfy consumer demand for mildly preserved, minimally processed, easily prepared and ready-to-eat fresher food with low spoilage potential (VERMEIREN, 1999; SONNEVELD, 2000).

1.2.3 Processing of milk

Fresh milk from animal sources must be a practically a natural sterile product, and all post-milking, handling and processing activities must maintain its nutritional value and prevent microbial contamination, off-odor development and physicochemical deterioration. Most cows are milked twice a day, although some farms milk three or four times per day. Milk is then immediately cooled from body temperature to below 40°F (5°C), and stored under refrigeration to be transported
by insulated tanker trucks at least every other day (Figure 1).

**Figure 1.** Shows the collection of milk from cows to the holding (storage) tanks and transfer through insulated tanker trucks to the processing units (http://rightmoves.tdtvictoria.org.au).

When milk is pumped into the tanker a sample is also collected for later lab analysis. On arrival to the milk processing units, it is checked to make sure it meets the standards for temperature, total acidity, flavor, odor, tanker cleanliness, and the absence of antibiotics. The raw milk is supposed to be processed within 72 h of receipt at the processing plant to prevent spoilage. Milk is such a nutritious food that numerous naturally occurring bacteria are always present. Thus, milk provides a favorable environment for the growth of microorganisms. Yeasts, moulds and a broad spectrum of bacteria can grow in milk, particularly at temperatures above 16°C.

Microbes can enter milk via the cow, air, feedstuffs, milk handling equipment and the milker (ALBRECHT and SUMNER, 1992). Once microorganisms get into the milk their numbers can increase rapidly. It is more effective to exclude microorganisms than to try to control microbial growth once they have entered the milk. Milking equipment is to be washed thoroughly before and after use; rinsing is not enough. Bacterial types commonly associated with milk are given in Table 1.

Milk is then pasteurized using a common process of heating the raw milk to kill all viable forms of bacteria that may be present, including pathogens. A pathogen is a species that could, if allowed to grow and multiply, cause serious health issues. Pasteurization is therefore applied to destroy the harmful bacteria but has adverse effects the sensory and nutritional quality of milk (Table 2).
Table 1. Bacterial types commonly associated with milk.

<table>
<thead>
<tr>
<th>Bacterial type</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em></td>
<td>Spoilage</td>
</tr>
<tr>
<td><em>Brucella</em></td>
<td>Pathogenic</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td>Pathogenic and spoilage</td>
</tr>
<tr>
<td><em>Staphylococci</em></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Pathogenic</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>Pathogenic</td>
</tr>
<tr>
<td><em>S. thermophilus</em></td>
<td>Acid fermentation</td>
</tr>
<tr>
<td><em>S. lactis</em></td>
<td>Acid fermentation</td>
</tr>
<tr>
<td><em>S. lactis-diacetyllactic</em></td>
<td>Flavour production</td>
</tr>
<tr>
<td><em>S. cremoris</em></td>
<td>Acid fermentation</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em></td>
<td>Acid fermentation</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Spoilage</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>Acid production</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>Acid production</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>Acid production</td>
</tr>
<tr>
<td><em>Propionibacterium</em></td>
<td>Acid production</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Pathogenic</td>
</tr>
</tbody>
</table>

Table 2. Adverse effects of pasteurization on nutritional quality of milk.

- Destroys PROBIOTICS
- Destroys ENZYMES
- Destroys VITAMIN D
- FATS are Deformed
- PROTEINS are Deformed
- IMMUNE complex
- CALCIUM bio-unavailable

It is well known that pasteurization process is not sterilization, which eliminates all viable forms of life including spores. After pasteurization, some harmless thermophilic bacteria may still survive the heating process and can cause the milk to become sour. Thus storing milk in a refrigerated temperature (4 to 5°C) is another way to keep the slow growth of these bacteria. Some bacteria do not cause spoilage,
but are actually added to milk or cream after pasteurization to make "fermented" products such as cheese, cottage cheese, yogurt, buttermilk, acidophilus milk, kefir, sour cream, etc. (FRAZIER & WESTHOFF, 1988; IAMFES, 1991).

### 1.2.4 Processing of water

One of the major concerns in protecting public health through drinking water utilities is aimed towards the reduction of waterborne disease and elimination of pathogens that are hazardous for human health. These pathogens, including bacteria, viral, and protozoan species, comprise a diverse group of organisms which serve as the etiological agents of waterborne disease (Table 3).

#### Table 3. Types and numbers of microorganisms typically found in untreated domestic wastewater.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Concentration (cells/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal coliforms</td>
<td>$10^{-4}$-$10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>Fecal Streptococci</td>
<td>$10^{-3}$-$10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>Enterococci</td>
<td>$10^{-2}$-$10^{-3}$</td>
<td>GANNON &amp; BUSSE, 1989</td>
</tr>
<tr>
<td>Shigella</td>
<td>Present a</td>
<td>OLIVIERI et al., 1977</td>
</tr>
<tr>
<td>Salmonella</td>
<td>$10^{-0}$-$10^{-2}$</td>
<td>GELDREICH et al., 1968</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>$10^{-1}$-$10^{-2}$</td>
<td>DUTKA &amp; RYBAKOWSKI 1978; DUTKA &amp; TOBIN, 1978</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>$10^{-1}$-$10^{-3}$</td>
<td>METCALF &amp; EDDY 1991;</td>
</tr>
<tr>
<td>Protozoan cysts</td>
<td>$10^{-1}$-$10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>Giardia cysts</td>
<td>$10^{-1}$-$10^{-2}$</td>
<td>JARROLL et al., 1981</td>
</tr>
<tr>
<td>Cryptosporidium cysts</td>
<td>$10^{-1}$-$10^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Helminth ova</td>
<td>$10^{-2}$-$10^{-1}$</td>
<td>BERMAN et al., 1984</td>
</tr>
<tr>
<td>Enteric virus</td>
<td>$10^{-1}$-$10^{-2}$</td>
<td>BERMAN et al., 1984</td>
</tr>
</tbody>
</table>

*a Results are usually reported as positive or negative rather than being quantified.*

But presence of toxic elements in drinking water is equally a major issue in the water treatment plants, for example fluorides in drinking water can be both beneficial as well as detrimental depending on their concentration and total amount ingested. Indian standards for drinking water recommend an acceptable fluoride...
concentration of 1.0 mg F/L and an allowable fluoride concentration of 1.5 mg F/L in potable waters (CPHEEO, 1984). Excess F in drinking water cause dental and/or skeletal fluorosis (SRIMURALI et al., 1998).

Treatment of drinking water is generally processed through 5 phases, i.e. Coagulation-Sedimentation-Filtration-Disinfection-Storage (Figure 2). Coagulation is a first treatment process that removes dirt and other particles that are suspended in water, using chemical aids that flocculate attracting the dirt particles. The formed “flocs” are heavy and eventually precipitate during the sedimentation step. The water is filtered through layers of sand, gravel, and charcoal to remove smaller particles and organic matter and is then placed in suitable tanks for disinfection using various disinfectants such as chlorine and ammonia (chloramination). Although such treatments of drinking water have gained increasing popularity, they have been associated with adverse effects on public health (HOFF, 1986).

![Figure 2. Flow diagram of a water treatment plant (http://water.epa.gov).](image)

The disinfection process may be affected by physicochemical factors, such as temperature and pH, turbidity, particle protection, and clumping of microorganisms, which may reduce the efficiency of most commonly used disinfectants (BERMAN et al., 1984). Resistance to disinfection may also vary among species and their different life-stages, as in the case of bacterial endospores or encysted forms of protozoa. Although coagulation, clarification, and filtration processes may dramatically reduce the microbial load, chemical disinfection is final and most efficient step to eliminate microorganisms in water.

Desalination, as one of the modern applications of membrane technologies, also shows potential for the cleaning of polluted drinking water and improvement of its quality. Finally, drinking water sources are much likely to be contaminated by
dangerous organic compounds that may derive from various treated and non-treated effluents. These pollutants are of high concern because their environmental fate, behavior and metabolites are not well known (Snyder et al., 2003; Jones et al., 2005).

Chapter 3 - Biopolymers

1.3.1 Biopolymers

Biopolymer is a term generally used to describe polymers that are biodegradable in nature, including synthetic and biologically produced polymers. Such polymers have properties that render them compatible for food uses, e.g. as packaging materials to protect products from moisture, increase shelf-life and make a product easier to distribute. Every biopolymer has its own material-specific properties, such as mechanical performance and barrier properties (e.g. oxygen permeability), that makes it suitable for specific applications. The most common uses of such polymers are as food packaging materials, drug delivery systems, medical implants, and various other industrial applications. The biopolymers can be classified as renewable (depending on the source origin) or synthetic (Hipolito et al., 2002; Nair & Laurencin, 2007; Siracusa et al., 2008).

Some of the important properties of biodegradable biomaterials are (Nair & Laurencin, 2007; Siracusa et al., 2008):

- Safety and non-toxicity
- Appropriate mechanical properties, barrier properties, and processibility for the indicated application
- Acceptable shelf-life
- Degradation time compatible with the indicated application
- Able to get metabolized in case of implant applications
- Chemical resistance
Biopolymers derived from renewable resources and produced by biological routes, are considered to be more important in this prime concern due to the fact that they are environmentally friendly and are generally (but not exclusively) biodegradable. This means that polymers that contribute to the protection of the environment also include biodegradable synthetic polymers. Biodegradation involves cleavage of hydrolytically or enzymatically sensitive bonds in the polymer (Figure 3). Depending on the mode of degradation, polymeric biomaterials can be further classified into hydrolytically or enzymatically degradable polymers. Most of the natural biopolymers undergo enzymatic degradation (NAIR & LAURENCIN, 2007) (Table 4).

**Table 4.**

Synthetic and natural polymers stand at opposite ends regarding their properties. For example, polyolefins are hydrophobic polymers, resistant to peroxidation, biodegradation, and hydrolysis, which is their main attribute in packaging. They can be made biodegradable by introduction of additives which promote oxo-biodegradation producing oxidation compounds assimilable by microorganisms. Natural polymers like cellulose and starch are hydrophilic, water wettable or swellable and biodegradable. Therefore, they are not technologically useful for food packaging where water resistance is required. Biodegradable aliphatic polyesters such as polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) stand between these two extremes (SIRACUSA et al., 2008).
Table 4: Biodegradable polymers (NAIR & LAURENCIN, 2007).

A. Hydrolytically degradable polymers as biomaterials

1. Poly(a-esters)
   - Polyglycolide
   - Polylactides
   - Poly(lactide-co-glycolide)
   - Polydioxanone
   - Polycaprolactone
   - Poly(trimethylene carbonate)

   Bacterial polyesters | Poly(3-hydroxybutyrate) (PHB)

2. Polyurethanes

3. Poly(ester amide)

4. Poly(ortho esters) (POEs) | POE-I, II, III, IV

5. Polyanhydrides
   - Poly[carboxy phenoxy propane]-{sebacic acid}] (PCPP-SA)

6. Poly(anhydride-co-imide)

7. Cross-linked polyanhydrides
   - Poly(sebacic acid) (PSA)/ poly(1-3 bis(p-carboxyphenoxy)propene) (PCPP); poly(1-6 bis(p-carboxy phenxoy)hexane) (PCPH)

8. Poly(propylene fumarate) (PPF)

9. Pseudo poly(amino acid)
   - Tyrosine derived polycarbonates and polyarylates; tyrosine containing poly(DTRPEG carbonate) and poly(DTR-PEG ether)

10. Poly(alkyl cyanoacrylates) (PCAs)

11. Polyphosphazenes

12. Polyphosphoesters
   - Polyphosphates; polyphosphonates

B. Enzymatically degradable polymers as biomaterials

1. Proteins and Poly(amino acids)
   - Collagen
     - Natural poly(amino acids): Cyanophycin; poly(e-l-lysine) and poly-g-glutamic acid (g-PGA) and esters
     - Synthetic poly(amino acids): Poly(L-glutamic acid) (L-PGA); Poly(aspartic acid) (PAA)
   - Elastin
   - Elastin-like peptides
To conclude, one of the fastest-growing materials sectors in the recent years has been the production of biopolymers from renewable resources. Global environmental concern, regarding the use of non-biodegradable petroleum based packaging materials has been encouraging researchers, industries and government to search for alternative materials made from natural biopolymers, originating from natural sources, such as starch, cellulose, chitin, etc., microbial polymers produced using renewable raw materials, mainly wastes, as well as biodegradable synthetic polymers such as, PHAs polyhydroxyalkanoates, polycaprolactone and PLA (ARUMUGAM et al., 1989; CHANDRA & RUSTGI, 1998; CIESLA & LACROIX, 2006; TIEN et al., 2000; NAIR & LAURENCIN, 2007).

Four types of currently important biopolymers will be discussed in this chapter: cellulose-based polymers, starch-based polymers, microbial biopolymers (polyhydroxyalkanoates) and synthetic biopolymers (polylactides). Emphasis will be given on nano/micro porous biopolymers and composite biopolymer materials suitable for food processing.

### 1.3.2 Cellulose-based biopolymers

Cellulose is one of the most abundant biopolymers on earth with versatile applications. The use of cellulose for making packing material is long established with cellophane being the most common one for packaging (Figure 4). Other cellulose polymer materials (e.g. cellulose acetate films) have also been commercially available for years but are lost market share to newer polymers such as polypropylene.

Cellulose plastics like cellulose acetate (CA), cellulose acetate propionate (CAP), and cellulose acetate butyrate (CAB) are thermoplastic materials produced by esterification of cellulose. The use of forest or crop cellulosic residues is currently attracting interest as a sustainable, environmental friendly substitute of fossil feedstocks in the plastics industry (RAY & BOUSMINA, 2005). Various raw materials such as cotton, recycled paper, wood cellulose, and sugarcane are being used in making cellulose ester biopolymers in powder form, which in the presence of different plasticizers and additives are extruded to various types of cellulosic plastics. The phthalate plasticizer, however, commonly used in commercial cellulose ester plastics, is now considered an environmental and health threat. Therefore, various research groups are trying to substitute it with eco-friendly plasticizers (e.g.
citrate and blends of citrate and derivatized vegetable oil) to yield more eco-friendly cellulose plastics (RAY & BOUSMINA, 2005).

![Figure 4. Cellophane production: Cellulose treatment with alkali and carbon disulfide to yield viscose which is then recovered into cellulose after extrusion and treatment with sulfuric acid and sodium sulfate (http://en.wikipedia.org/wiki/Cellophane).](image)

Cellulose-based biopolymers have also been used for ropes, sails, paper, and timber for housing and many other applications (EICHHORN et al., 2010; EICHHORN, 2011). Native cellulose is found in macroscopic fibers which exhibit a hierarchical structure composed of smaller and mechanically stronger entities (fibrils). These fibrils display high stiffness and therefore are suitable for the reinforcement of nanocomposite materials (POMMET et al., 2008) with a wide range of applications such as nanopapers (HENRIKSSON et al., 2007) stable membranes, semi structural applications, (HUANG et al., 1998), liquid electrolyte Li-ion batteries (SCROSATI, 2007), flexible energy storage devices and supercapacitors (PUSHPARAJ et al., 2007), etc. In addition, by grafting DNA to the surface of cellulose nanofibres (cellulose/DNA hybrid nanomaterials) it is also possible to utilize self-assembly methods to generate new forms of composite biomaterials with potential applications including the scaffolding for tissue engineering applications (MANGALAM et al., 2009). Also, the use of combined nanocomposites based on cellulose nanofibres coated with SnO$_2$ have been proposed to design more stable membranes (HUANG et al., 1998). Finally, since bacterial cellulose (BC) was discovered, it has been the subject of increasing attention owing to its superior properties and wide scope of possible applications (CHOI et al., 2012).

In conclusion, cellulose is considered to be a renewable raw material resource for the increasing demands for environmentally friendly and biocompatible products (KALPAN, 1998). The physicochemical properties of cellulose, and recent advances on cellulose-based materials used for plastics, nanotechnology (biopolymers, nanomaterials, composites, etc.), foods (promotion of fermentation processes), and other applications are highlighted and discussed below, as cellulose is the main biopolymer used in this thesis.
1.3.3 Starch-based biopolymers

Starch is a natural polymer found in the form of granules in plant tissues from where it can be extracted in large quantities. It is the main storage carbohydrate in plants, and can be obtained from potatoes, maize, wheat, tapioca and similar other sources. Starch is considered as one of the most promising renewable polymeric materials because it is readily available and can be modified in many ways to produce a variety of cost effective end products, among which thermoplastics suitable for conventional plastic forming processes (RAY & BOUSMINA, 2005).

Starch is composed of a mixture of two α-D-Glucose polymers, the linear polysaccharide amylose (10–20%; 200–20000 glucose units; helical structure) and the highly branched polysaccharide amylopectin (80–90%; up to two million glucose units). Waxy starches contain almost 100% amylopectin. In the native form of starch, these two polymers are organized in granules consisting of alternating complex semicrystalline and amorphous layers (Figure 5) (RAY & BOUSMINA, 2005; SIRACUSA et al., 2008).

Figure 5. Starch: amylose, amylopectin and starch granule (http://www1.lsbu.ac.uk; http://en.wikipedia.org/wiki/Starch).
Starch is completely biodegradable in soil and water and it promotes the biodegradability of non degradable plastics when blended together. The starch remains in granular form in the plastic matrix and thus may act as filler (RAY & BOUSMINA, 2005; SIRACUSA et al., 2008). Starch, however, shows no plastic behavior, no adequate mechanical properties and it thermally degrades at around 260°C. When extruded with plasticizers it can become a thermoplastic, mouldable material with excellent oxygen barrier properties, but it is extremely sensitive to moisture and biodegrades rapidly. Depending on starch percentage and other additives, the properties of these materials can vary significantly. Starch can also be transformed into a foamed material replacing the polystyrene foam as packaging material. Starch combined with PHA polymers can give excellent packaging films (SIRACUSA et al., 2008). Hydrophilic compounds, such as polyols, as well as sugars, surfactants, amino acids and fatty acids are used in starch films to improve their mechanical and barrier properties. However, a limiting factor for the development of starch-based materials is the brittle nature of blends containing high concentrations of starch due to starch re-crystallization (retrogradation) during long-term storage. Therefore, an ideal plasticizer should suppress retrogradation and retain flexibility of thermoplastic starch products during aging (VIEIRA et al., 2011).

1.3.4 Polyhydroxyalkanoates (PHAs)

Bacterial polyhydroxyalkanoates (PHAs) are a family of polymers that serve as a carbon/energy store in more than 300 species of Gram(+) and Gram(-) bacteria and many archaea (Figure 6). They are biosynthesized as insoluble cytoplasmic inclusions in the presence of excess carbon source when other nutrients such as O₂, P, or N are limited. They are able to be stored at high concentrations in cells as they do not substantially affect its osmotic state. PHAs are piezoelectric and perfectly isotactic/optically active (only (R)-configuration), hydrophobic, water-insoluble, inert and indefinitely stable in air, non-toxic and completely biodegradable. They are also thermoplastic and/or elastomeric and have much higher resistance to UV degradation than polypropylene but are less solvent resistant (LAYCOCK et al., 2013).

More than 150 different monomer units have been identified in biological PHAs, giving them very broad polymer properties. PHAs can be divided into two main groups, the short-chain-length (3-5 C monomer units) and the medium-chain-length (6-18 C monomer units). The most common PHAs are the polyhydroxybutyrates (PHBs), poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] (Figure 7). These polymers have comparable mechanical properties with polypropylene and polyethylene, although they are more brittle due to high crystallinity and relatively large spherulites (LAYCOCK et al., 2013; SIRACUSA et al., 2008; LIM et al., 2013).
1. Introduction

**Figure 6.** Overview of PHA synthesis: (A) chain polymerization catalyzed by enzymes; (B) a PHA granule with associated proteins; (C) α and β forms of the PHB polymer chain; (D) semi-crystalline polymer structure; (E) AFM image of PHBV film; (F) final plastic products (LAYCOCK et al., 2013).

They exhibit a high melting point (173-180°C) and a glass transition temperature of 1-5°C. Both PLA and PHB are biodegradable polyesters with similar applications in food industries as packaging materials or in several other industrial sectors due to their biocompatibility, biodegradability and sustainability. PHBs are also used as medical implants due to their non-toxic and biodegradable character. In pure form or
as composites with other materials they can be used as sutures, repair patches, orthopedic pins, adhesion barriers, nerve guides and bone marrow scaffolds. Alone, or in combination with synthetic plastic or starch, PHAs can give excellent packaging films. Regardless of the fact that PHBs present versatile applications, their production and sales are not widely developed, due to the high production costs (ABDELWAHAB et al., 2012; VERLINDEN et al., 2007; SIRACUSA et al., 2008).

The simplest form for PHA production is to use pure cultures in 2-stage batch processes. In the 1st (growth) stage a sterile medium containing trace elements, a suitable carbon source and nutrients is inoculated. In the 2nd stage, an essential nutrient (such as N, P or O₂) is deliberately limited for PHA accumulation to occur. When the medium is limited with carbon source, depolymerases degrade the stored PHAs and the degraded products are utilized for cell metabolism (OJUMU et al., 2004). Thus, the final product depends on the type of carbon feedstock, the metabolic pathways of the specific bacteria, and the substrate specificities of the enzymes involved. Recently, PHA production using mixed cultures or genetically modified species have been proposed as a means of lowering production costs (LAYCOCK et al., 2013; PANDIAN et al., 2009). These polymers can also be produced by bacterial fermentation of sugar and lipid containing waste substrates. Different types of wastes as carbon sources have been evaluated to produce several PHA polymers with different physical and mechanical properties, like flexibility, tensile strength, melting viscosity but without any commercial application so far. Alone or in combination with synthetic plastic or starch PHAs can give excellent packaging films (SIRACUSA et al., 2008; PANDIAN et al., 2009).

1.3.5 Polylactides (PLA)

Polylactide (PLA) is a biodegradable synthetic aliphatic polyester which has been widely studied for a variety of applications in growing technologies such as medical implants, drug delivery, and packaging materials. The lactic acid monomer (2-hydroxypropionic acid) can be easily obtained by a biotechnological process, usually based on bacterial strains (mainly Lactobacillus), which produce pure enantiomers (mainly L-lactic acid). Inexpensive renewable raw materials can be used, which can be either in the form of pure sugar (glucose, sucrose, lactose media) or sugar-containing materials such as molasses, whey, sugarcane, sugar beet, starchy materials (cassava, potato, cereals), bagasses, etc. (GUPTA & KUMAR, 2005; HIPOLITO et al., 2002 & 2012).

In order to meet the different performance requirements, PLA can be synthesized by various methods using different catalysts (Figure 8). Lactic acid obtained by chemical synthesis is racemic (both D- and L-enantiomers are produced). Generally, there are four methods used for the synthesis of PLA (GUPTA & KUMAR, 2005):

- **Direct polycondensation polymerization** (lactic acid is polymerized in the presence of catalyst at reduced pressure yielding low molecular weight polymers)
- **Azeotropic condensation polymerization** (high molecular weight PLA is synthesized)
1. Introduction

- **Solid state polymerization** (involving heating of semi-crystalline prepolymer of relatively low molecular weight, up to temperatures below the melting point with simultaneous removal of by-products by reduced pressure or with an inert gas carrier)

- **Ring opening polymerization** [PLAs are made by polymerization of their respective cyclic dimmers, i.e. lactides (six member cyclic 3,6-dimethyl-1,4-dioxane-2,5-dione dimmers). Lactides are prepared from thermal cracking of low molecular weight PLA oligomer at high temperature/low pressure in the presence of catalyst]

**Figure 8.** Main methods for PLA production (GUPTA & KUMAR, 2005.)

However, recent efforts involve the production of PLA by direct fermentation of metabolically engineered species (e.g. *Escherichia coli*) equipped with evolved enzymes (e.g. propionate CoA-Transferase and PHA synthase) (JUNG & LEE, 2010).

PLA and its copolymers are promising biomass derived polymers due to their excellent properties including biocompatibility, compostability and low toxicity to humans, which also makes them suitable for household products, commercial plastics, and biomedical materials (DRUMRIGHT et al., 2000; SODERGARD & SLOT, 2002; VINK et al., 2003). PLA is a high strength, high modulus thermoplastic, which can be easily processed by conventional plastic processing techniques (injection and blow moulding, thermoforming and extrusion). For large-scale production, the polymer must possess good thermal stability to prevent degradation and maintain its molecular weight and properties. PLA degradation takes place by simple hydrolysis of the ester bond and depends on time, temperature, impurities, and catalyst concentration. The PLA degradation products are non-toxic (GUPTA & KUMAR, 2005).

A large number of investigations have been carried out on PLA and its copolymers in biomedical applications for medical implants (SCHWACH & VERTB, 1999), drug delivery systems (LEO et al., 2004), scaffold of tissue and organ regeneration (SEALA et al., 2001), films or foils for wound treatment (KRICHELDORF, 2001), agricultural...
applications like mulch films, slow release of pesticides and fertilizers, etc. (GUPTA & KUMAR, 2005). The main limitations of PLA towards wider industrial application (such as packaging), is their poor thermal resistance and limited gas barrier properties (SINHA et al., 2005). Nevertheless, these drawbacks can be overcome by the development of nanocomposites by addition of nanoparticles (KIERSNOWSKI et al., 2004; RAY & BOUSMINA, 2005). PLA based nanocomposites have higher rates biodegradation in compost by the addition of nanoclays due to the high relative hydrophilicity of the clays, which allows easier penetration of water and activation of hydrolytic degradation mechanisms (PAUL et al., 2005). Studies on nanosize and microsize clay effects on PLA thermal degradation kinetics, showed environmental sustainability and performance (ZHOU & XANTHOS, 2009).

Other applications of PLA in the food industries are: (1) as biodegradable packaging materials for the storage of wine in place of polyethyleneterephthalate and glass (PATTI et al., 2010); (2) as depth filter microfiltration membranes acting as pre-filters to decrease microbial load in industrial wastes in both biochemical and food industries (TANAKA et al., 2012); (3) as packaging films, stable during high pressure pasteurization and sterilization, for tap water, wild carrots, carrot puree and carrot juice (SANSANE et al., 2012).

The use of natural fibers, mainly of plant origin, as alternatives to glass or synthetic fibers has been of much current research interest, due to environmental as well as health and safety advantages (EICHHORN et al., 2001; OKSMANAY et al., 2003). Natural fibers have been used for reinforcement of PLA plastics in order to sustain higher mechanical properties compared to pure PLA (PLACKETT et al., 2003; NISHINO et al., 2003). Some minerals (RAY et al., 2005; RAY et al., 2003) and polysaccharides (e.g. starch and chitosan) (YEW et al., 2005) have also been proposed for reinforcement of PLA composites.

To conclude, among various other kinds of degradable polymers, PLA is currently a most promising material with the brightest development prospect, and is considered to be the “green” eco-friendly material. PLA is a sustainable alternative to petrochemical-derived products, since lactides from which it is produced, can be produced on mass scale by microbial fermentation of agro-industrial by-products and wastes. Lactic acid is the most widely occurring hydroxycarboxylic acid, having a prime position due to its versatile applications in food, pharmaceutical, textile, leather and chemical industries (VICKROY, 1985; NAMPOOTHIRI et al., 2010; JOHN et al., 2006a; GUPTA & KUMAR, 2005).

### 1.3.6 Nano/micro fabrication of biopolymers

With the advancement of civilization and technologies for better living, the importance of biopolymers and their applications in various fields of life, particularly food and medical technologies, has been made clear and realistic. Though biopolymer technologies are growing very fast, still there is need for further research to develop improved materials for specified purposes. In this regard, in the last few decades, there has been significant progress in one-dimensional nanostructures with nanoscale and molecular scale properties that can satisfy the demands of the 21st century, having a great impact on fundamental research and industrial applications.
in nano and molecular electronics, nanodevices, nanocomposites, nanobiotechnology and medicine. Such structures are the carbon nanotubes, inorganic semiconducting and metallic nanotubes/wires, conjugated polymer nanofibers/tubes, etc. (LONG et al., 2011).

Advances in micro- and nano-fabrication technologies have allowed the successful fabrication of a variety of implantable and drug delivery devices based on silicon, glass, or plastic materials. However, precise micro- and nano-fabrication of biopolymers still remains an important challenge. Replication technologies, which have proven useful in this respect, are based on replication of a microfabricated mold tool with inverse geometry of the desired polymer structure. This expensive microfabrication step is necessary for the initial fabrication of the master structure, which then can be replicated many times into the polymer substrate (LU & CHEN, 2004).

The structure-building elements in most foods are colloidal and are built up as a result of self assembling of nano/micro sized molecules into particles. The ability to control the length scales of these particles of macromolecules or other minor components in a food matrix will become an integral part of food product design. Therefore, the next wave of food innovation will regard a shift of focus from macroscopic to meso- and nano-scale properties in order to successively control the hierarchical configurations in food and their functionality (SANGUANSRI & AUGUSTIN, 2006).

Chapter 4 - Composite materials

1.4.1 Composites

As highlighted above, most biodegradable polymers have excellent properties comparable to many petroleum-based plastics, but some of their disadvantages include brittleness, low heat distortion temperature, low gas permeability, and low melt viscosity. These properties restrict their use for further processing in a wide range of applications. To solve these problems, modification through innovative technologies, such reinforcement to prepare composites (especially nanocomposites), is a formidable task for the material scientist (RAY et al., 2005;
1. Introduction

HANS et al., 2002; RAY & OKAMOTO, 2003). “Composites are a class of materials that comprise two or more distinct components with significantly different physical and/or chemical properties” (MIAO & HAMAD, 2013). The resulting material can have enhanced mechanical performance and/or new properties and functionalities (e.g. barrier properties). Usually, a composite contains a strong and stiff component, the “reinforcement”, embedded in a softer constituent, the “matrix”. Many natural materials are composites, such as wood fibers (cellulose microfibrils embedded in an amorphous matrix of lignin, hemicelluloses and small amounts of pectins and proteins), bone and teeth (inorganic crystals in a matrix of collagen), etc. Depending on the type of matrix, composites can be classified into 3 main categories (MIAO & HAMAD, 2013):

(1) Polymer Matrix Composites (PMCs)
(2) Metal Matrix Composites (MMCs)
(3) Ceramic Matrix Composites (CMCs)

The majority of artificial composites contain phenolic and epoxy resins, rubber, thermoplastic polymers [poly(ethylene), poly(propylene), poly(vinyl chloride), poly(ethylene oxide)], etc. However, due to growing concerns for sustainability and environmental protection, efforts have been made to produce biodegradable composites based on cellulose, starch, PLA, PHB, poly(hydroxyoctanoate), etc. Reinforcements in composites can be classified based on their structure as woven, continuous fibers, short fibers and particles, and based on the morphology (size) as macroscopic, microscopic or nanoscopic (MIAO & HAMAD, 2013).

1.4.1.1 Nanocomposites

Nanocomposites are a relatively new generation of materials consisting of polymers reinforced with particles (nanofillers) that have at least one nano-scale dimension (1-100 nm). The reinforcement can be either organic or inorganic resulting in composites with enormous surface area and superior thermal, barrier, or mechanical properties. The advantage of nanofillers is that due to their low size they are miscible with the polymer matrix, allowing unique synergisms between the blended materials. Such polymer/nanoparticle mixtures (nanocomposites) have been a rapidly growing field of research in the last few decades for developing a wide range of materials with exceptional properties suitable for a wide range of applications (BIKIARIS, 2013; SAIN & OKSMAN, 2005; MIAO & HAMAD, 2013; GARCES et al., 2000; JORDEN et al., 2005; MATSUMURA et al., 2000; NISHINO & ARIMOTO, 2007; GINDL & KECKES, 2005; SOYKEABKAEW et al., 2009; KRISTO & BILIADERIS, 2007). Nanofillers can be classified into three main categories depending on their dimensions (BIKIARIS, 2013):

(a) Nanoparticles (isodimensional nanoparticles);
(b) Nanotubes (or nanofibers, whiskers, nanorods (two dimensions in the nanometer scale and the third is larger);
(c) Nanolayers (in the form of nanolayers/nanosheets with few nanometers thick). Generally
Food related applications of nanotechnologies offer a wide range of benefits to the consumer, although the level of nanotechnology applications in these sectors is still at R&D or near-market stages (CHAUDHRY & CASTLE, 2011):

- possible reduction of preservatives, salt, fat and surfactants in food products;
- development of new or improved tastes, textures and mouth sensations;
- improved uptake, absorption, and bioavailability of nutrients;
- new lightweight and stronger packaging materials to keep food safe during transportation, increase shelf-life and reduce microbial spoilage;
- antibacterial nanocoatings on food surfaces to maintain hygiene during food processing;
- 'Smart' labels to help protect safety and authenticity of products in the supply chain,

1.4.2 Natural fiber/cellulose composites

Natural fiber reinforced polymers constitute an important branch in the field of composite materials. Natural fibers derive from different sources (wood, straw, pulp, cotton, bagasse, corncobs, etc.) and have different composition depending on the origin. They are mainly made of cellulose, hemicelluloses, lignin and pectin, with a small quantity of extractives (XIE et al., 2010).

Cellulose is one of the most abundant biopolymers in nature, which has now been acknowledged for exploitation due to its biocompatibility for numerous food and other applications. Wood is the major source of cellulosic fibers, especially for the pulp and paper industry, although a variety of other agri-fibers are available. Cellulose microfibrils normally have a diameter of 5–50 nm and lengths of thousands of nanometers. Natural fibers are composed of only 55-65% depending on the plant species and a number of other environmental factors. They can serve as reinforcement to improve the strength and stiffness, and also to reduce the weight of the resulting composite materials (OKSMANA et al., 2003).

Specifically, cellulosic composites have several advantages: (1) cellulose is biodegradable and CO$_2$ neutral; (2) is abundant and renewable resource worldwide; (3) is found in a wide variety of fibers; (4) is a low cost raw material; (5) has high specific strength and modulus (maximum macroscopic Young's modulus of natural plant cellulose fibers is 128 GPa); (6) has high sound damping performance due to the hollow structure of fibers; (7) has low density; (8) has relatively reactive surface susceptible to modification. However, composites reinforced with natural fibers are not widely used in industrial applications mainly due to drawbacks regarding polarity, hygroscopicity and hydrophilicity, non-compatibility with non-polar/hydrophobic thermoplastics, limiting processing temperature range for compounding with major engineering plastics, etc. (MIAO & HAMAD, 2013; PANDEY et al., 2010; NOVAK et al., 1997; SANCHEZ-GARCIA et al., 2008; MONTEIRO et al., 2012).

Therefore, treatment of natural fibers is beneficial in order to improve their water resistance, enhance the wettability by non-polar polymers and promote interfacial
adhesion. Physical treatments (e.g. electronic discharge) may be used to increase the compatibility of the treated fiber surface with the polymer matrix. Chemical modification can permanently alter the nature of the fiber surface, e.g. by grafting polymers onto the fibers, crosslinking of the fiber cell walls, or by using coupling agents (chemical bridges between the reinforcement and matrix) (XIE et al., 2010).

Such composite materials have been used for long in everyday practical applications like when grass and straw were used to reinforce mud bricks (TAIB, 1998). Plant-based natural fibers like flax (OKSMANA et al., 2003), jute (PLACKETT et al., 2003), sisal (JOSEPH et al., 2003) and kenaf (NISHINO, et al., 2003) have been frequently utilized and studied due to their natural abundance, cost effectiveness, world annual production and a wide range of properties depending on the plant source.

1.4.2.1 Nanocellulose

About 36 individual cellulose molecules are brought together by biomass into larger units known as elementary fibrils or microfibrils, which are packed into larger units called microfibrillated cellulose. The latter is in turn assembled into cellulose fibers, which are presented schematically in Figure 9. The diameter of microfibrils is about 5 nm whereas the microfibrillated cellulose has diameters ranging from 20 to 50 nm. The microfibrils are several micrometers in length. Each microfibril is made up of cellulose crystals linked along the microfibril axis by disordered amorphous domains. The ordered regions are cellulose chains packed together by a strong network of hydrogen bonds (HABIBI et al., 2010; AZIZI et al., 2005). The two main types of nanocellulose are: (i) cellulose nanocrystals and (ii) cellulose microfibrils.

There is yet another source of nanocellulose, bacterial cellulose, which is secreted extracellularly by specific bacteria, mainly by Gluconacetobacter strains, in the form of cellulose nanofibers (KLEMM et al, 2009, 2006; SIRO & PLACKETT, 2003). For example, G. xylinus secretes microfibrillated cellulose having a width of about 3.5 nm (KOSE et al., 2011). Bacterial cellulose has unique fibrillar nanostructure and excellent physical and mechanical properties such as high porosity, high elastic modulus (GUHADOS et al, 2005; SAHEB & JOG, 1999; SIRO & PLACKETT, 2010), and
1. Introduction

High crystallinity (up to 84–89%) (CZAJA & BROWN, 2004). Nowadays, bacterial cellulose is the subject of investigation on numerous applications such as biomedical (CZAJA et al., 2007; KLEMM et al., 2006; LEE et al., 2005), reinforcement in nanocomposites (JUNTARO et al., 2008; NAKAGAITO & YANO, 2003; NOGI & YANO, 2008), electronic papers (SHAH & BROWN, 2004), and fuel cell membranes (EVANS et al., 2003).

1.4.2 Other biopolymer-based composites

1.4.2.1 Biopolymers

Various biopolymers from renewable resources have been found suitable for environmentally friendly and biodegradable composites production, such as soy-oil based epoxy, starch based polymers, polycaprolactone (PCL), PHAs (PHB, PHV, PHBV), PLA, polyglycolate (PGA), poly(butylene succinate) (PBSu), poly(ethylene succinate) (PESu), poly(propylene adipate) (PPAd), etc. These biopolymers have been evaluated in plain form, in combination (e.g. PLA/PHB) and in unfilled state. The reinforcement (e.g. with natural fibers) gives to these polymer composites better mechanical properties (stiffness, strength, and toughness) as well as, a low cost alternatives to composites reinforced with glass synthetic fibers (LOUREIRO et al., 2014; AVELLA et al., 2000; BIKIARIS, 2013).

Polymer blending is a convenient industrial process since it provides materials to cover a large spectrum of specific needs, excluding synthetic stages. Such materials are easily processable and preserve the major properties of the contained moieties. The main reason for blending is economy, sustained by (1) improving polymer performance by blending it with a low cost polymeric material, (2) obtaining materials with specific properties, and (3) ability to recycle plastic waste (AVELLA et al., 2000).

Blending and filling techniques, especially the addition of nanofillers, can further improve the performance and multifunctionality of biopolymer composites. The most commonly used nanofillers in polymer composites are: layered silicates (such as montmorillonite), nanotubes (mainly carbon nanotubes), fullerenes, carbon black, nanodiamonds, SiO$_2$, metal oxides (e.g. TiO$_2$, Fe$_2$O$_3$, Al$_2$O$_3$), metal nanoparticles (e.g., Au, Ag), polyhedral oligomeric silsesquioxane (POSS), semiconductors (e.g. PbS, CdS), etc. (BIKIARIS, 2013).

1.4.2.2 Lignin

Lignin is an amorphous phenolic cell wall polymer closely linked to cellulose and hemicelluloses in lignocellulosic biomass, and is the second most abundant biopolymer on earth. Its valorization represents an important challenge because, despite being one of the most abundant natural polymers, after cellulose and chitin, most of the biomass lignin (as a by-product of the bioethanol fermentative production process) is burned or landfill disposed. Only a minor amount is employed
for the as a raw material in the production of added value materials, such as improved composites after blending with thermoset and thermoplastic polymers (ANGELINI et al., 2014). Examples are the use of lignin in copolymers doped by carbon nanotubes for chemical sensors applications or as substitutes of bisphenol-A in epoxy resins. A number of studies also report the effect of lignin in blends with polyvinyl acetate, polyethylene and polypropylene. When blended with biodegradable aromatic and aliphatic polyesters, it increases the thermal stability and elastic moduli of the resulting composites. In this frame, PHAs are an attractive family of naturally occurring polyesters, with PHB (P3HB) being the most important member as a biodegradable, biocompatible thermoplastic polymer, comparable to polypropylene. However, PHB is expensive and brittle and undergoes thermal degradation and depolymerization at temperatures close to its melting point. As a result, the preparation of PHB composites by blending with other polymers and fillers (e.g. lignin) has been investigated aiming at producing materials with improved properties (ANGELINI et al., 2014).

1.4.2.3 Hydrocolloids

Food-grade biodegradable materials such as polysaccharides, proteins and lipids are edible and have attracted considerable interest in recent years due to their potential abilities to act as food contact edible films and/or coatings replacing conventional plastics due to their sustainable nature, compostability, environmentally-friendly character and compatibility with food application (NUR HANANI et al., 2014).

*Gelatin*

Gelatin is a hydrocolloid material widely used in the food, pharmaceutical, cosmetic and photographic industries due to its unique functional properties. It is derived from the fibrous insoluble protein collagen (by chemical denaturation) and is obtained from bones, skin and connective tissue generated as animal processing waste. In the food industry, gelatin is used as gelling agent, stabilizer, texturizer and emulsifier for bakery, dairy, beverages and confectionary. In the pharmaceutical sector it is used for capsules, plasma expanders, ointments, wound dressing and emulsions. Gelatin from different sources has different physicochemical properties, due to different amino acid composition, which are responsible for the varying characteristics. Packaging films can be successfully produced from all gelatin sources and the behavior and their characteristics can be modified by incorporation of other ingredients to produce composites with enhanced physical and mechanical properties. Single gelatin films tend to swell or dissolve when in contact with foods with high moisture contents due to their high hygroscopicity. Blending two different polymers (e.g. protein with polysaccharide or lipid or other synthetic/natural polymer) can produce improved composite films, e.g. with limited moisture migration, improved mechanical properties, etc. (NUR HANANI et al., 2014).

Nanoparticles (e.g. nanoclays) have also been shown to offer novel characteristics (such as improved barrier properties and tensile strength, decreased gelatin solubility, and antimicrobial activity against food borne pathogens) when incorporated in gelatin-based films (NUR HANANI et al., 2014).
**Alginates**

Alginate is linear naturally occurring polysaccharide copolymer, consisting of (1→4) β-D-mannuronic acid and α-L-guluronic acid. Alginate is a popular ingredient for food products because of its many unique colloidal properties, including thickening, stabilizing, suspending, film forming, gel producing, and emulsion stabilizing (HARPER et al., 2015). Alginate is found in seaweeds and typically extracted from brown algae (Phaeophyceae) including Laminaria hyperborea, L. digitata, L. japonica, Ascophyllum nodosum, and Macrocystis pyrifera through alkali treatment (VENKATESAN et al., 2015).

Alginate is able to form gels in the presence of certain divalent metal cations (mainly calcium). As in the case of gelatin, there is growing interest in using alginates for biodegradable packaging film production. Researchers have explored both the mechanical and barrier properties of alginate films, the effect of addition of various proteins (soy, whey, and gelatin) and polysaccharides (kappa-carrageenan, pectin, pullulan, cellulose, carboxy-methyl cellulose, modified or unmodified potato starch, sago starch, gellan gum, etc.). In certain cases, the alginate-carbohydrate composite films had superior properties compared to their individual component films (HARPER et al., 2015).

Alginate is also gaining interest for biomedical applications, particularly in bone tissue engineering due to its biocompatibility and gel forming properties. Several composites have been evaluated, such as alginate-polymer (polyethylene glycol, chitosan, etc.), alginate-protein (collagen and gelatin), alginate-ceramic, alginate-bioglass, alginate-biosilica, alginate-bone morphogenetic protein-2 and RGD peptides. These composites showed improved properties for biomedical applications (e.g. bone tissue regeneration) in terms of porosity, mechanical strength, cell adhesion, biocompatibility, cell proliferation, etc. (VENKATESAN et al., 2015).

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**Chapter 5 - Cellulose as a bioprocess advancing tool**

**1.5.1 Plant cellulose**

Cellulose is one of the most abundant organic compounds derived from biomass with an estimated annual production of $1.5 \times 10^{12}$ tons available worldwide and is considered to be an almost inexhaustible source of raw materials (KLEMM et al., 2005; CAO et al., 2009). Of this, only about $6 \times 10^9$ tons are processed by industries as an enormous underutilized energy resource, and for the production of paper, textiles, panel products, chemicals, and other industrial products (SIMON et al., 1998).

Although cellulose is the main building material of wood, there are other major sources such as plant fibers (cotton, hemp, flax, etc.), marine animals (tunicate), algae, fungi, invertebrates, and bacteria. In plants, it is the major constituent of cell
walls and its content depends on the plant species, growing environment, and maturity. Generally, the cellulose content in lignocellulosic plant biomass is 23–53\% on a dry-weight (dw) basis, less than that in cotton, which is almost made of pure fibrous cellulose (KNAUF et al., 2004). In most straw species, approximately 35–45\% of the dry substance is cellulose. The cellulose content of rice straw is 36.5 \% (dw). Plant cell walls provide strength and support for plants, limit the size and shape of cells, and act as a barrier to potential pathogens. Cellulose exists as microfibrils of high length and varying degrees of crystallinity and is embedded in a complex matrix composed of hemicelluloses, lignins, and other carbohydrate polymers, as described above (HANLEY et al., 1997; YU et al., 2005).

Cellulose is a linear homopolysaccharide with a high degree of polymerization, consisting of glucose units linked with \(\beta(1\rightarrow4)\) glycosidic bonds. Its linear structure allows intra- and intermolecular hydrogen bonding resulting in aggregation of cellulose chains into elementary tightly packed crystalline microfibrils (2–20 nm diameter) (Figure 10), which are covered and interlocked by hemicelluloses (MORAN-MIRABAL, 2013; XU et al., 2013; JORGENSEN et al., 2007).

Figure 10. Details of the cellulosic fiber and molecule structure (http://bio1151.nicerweb.com/Locked/media/ch05/cellulose.html).
The ability of cellulose chains to form hydrogen bonds confers upon cellulose its most important properties: (i) the microfibrillated structure, (ii) the hierarchical organization (alternating crystalline and amorphous regions), and (iii) the highly cohesive nature (glass transition temperature higher than its degradation temperature). Cellulose can be found in 4 types (I, II, III and IV), of which Cellulose I, the native cellulose, has two crystalline modifications: cellulose I\( \beta \) found mainly in plant cell walls, and cellulose I\( \alpha \) found in some microorganisms (Figure 11) (XU et al., 2013). Cellulose II, or regenerated cellulose, emerges after recrystallization with aqueous sodium hydroxide.

It is the most stable crystalline form (AULIN, 2009; SIQUEIRA et al., 2010). The major distinction between these two forms, lies in the layout of their atoms: Cellulose II has antiparallel packing, whereas the chains in cellulose I run in a parallel direction (AULIN, 2009).

The structure of lignocellulosic biomass, analytical techniques available for its analysis, as well as the effect of pretreatment methods, used for biofuel production, on the structure of biomass components have been widely reviewed and discussed (MORAN-MIRABAL, 2013; XU et al., 2013; LUPOI et al., 2014; SOREK et al., 2014; PINGALI et al., 2014).

1.5.1.1 Isolation of cellulose fibers

The isolation of highly pure cellulose has been the subject of extensive research for many years because of the complexity of cell wall structure (BRENDEL et al., 2000; SUN et al., 1998). The combination of chemical and mechanical treatments is necessary for the dissolution of lignin, hemicelluloses, and other non-cellulosic
substances. A protocol based on acidified sodium chlorite is frequently applied to delignify cellulosic materials as an initial step to isolate cellulose (LOADER et al., 1997). Treatment with chlorite can remove almost all of the lignin content and the following isolation of cellulose can be performed at room temperature with alkali extraction. Pulping and bleaching are common techniques used in paper industries to remove lignin, hemicelluloses, and other non-cellulosic substances and obtain pulp fiber with high cellulose purity and brightness, using chemical and mechanical processing. Chemical pulping includes soda, sulfate, and sulfite processing. In these procedures, NaOH, Na₂S H₂SO₄, Na₂SO₃, NaHSO₃ and/or SO₂ are the major active chemicals for impregnation and delignification. The presence of lignin in isolated cellulose fibers after delignification affects the structure and properties of the fibers. Fibers with high amounts of lignin are coarse, stiff, and have a brownish color. To obtain fibers that are relatively free of bound lignin, chemical bleaching is usually necessary to follow the delignification process.

1.5.1.2 Lignin

Lignin consists of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units derived from the polymerization of p-coumaryl, coniferyl, and sinapyl alcohols (monolignols), respectively (Figure 12). Lignin is physically and chemically connected with hemicelluloses, and embeds the cellulose portion offering waterproofing and protection against microbial and chemical degradation. In addition to the three main monolignols, lignin contains traces of units from incomplete monolignol biosynthesis and incorporates various other phenylpropanoid units, such as hydroxycinnamyl aldehydes, acetates, p-coumarates, p-hydroxybenzoates and tyramine ferulate (XU et al, 2013; JORGENSEN et al., 2007; VAN DYK & PLETSCHKE, 2012; SEDEROFF et al., 1999; BOERJAN et al., 2003).

The complexity and heterogeneity of the polymer depend on the relative proportions of the three monolignol units as well as the different types of interknit linkages (CAMPBELL & SEDEROFF, 1996; MONTIES, 1998). For example, lignin from gymnosperms consists mainly of G units and low levels of H, whereas lignin from angiosperms is predominantly made up of G and S units with traces of H. Lignin rich in G units, such as in gymnosperms, has relatively more carbon bonds than lignin rich in S units because the aromatic C5 position of G units is free to make linkages.
1.5.2 Bacterial cellulose

Bacterial cellulose (BC) is another form of microfibrillated cellulose that differs in structure and mechanical properties from that of plant cellulose, regardless of their identical chemical composition. Since BC was discovered, it has gained increasing attention due to its superior properties, such as high porosity, water holding capacity, elasticity, mechanical strength, and thermal stability, that have been attributed to its micro/nanoscale 3D-network structure formed by inter- and intra-fibril hydrogen bonding. Because of its superior properties and biocompatibility, BC has potential for a wide variety of applications, such as optically transparent composites, dietary foods, medical wound dressings, separation media, fuel cells, optoelectronics, etc. (CHOI et al., 2012; BROWN & LABORIE, 2007).

BC is synthesized by bacteria belonging to the genera *Acetobacter*, *Rhizobium*, *Agrobacterium*, and *Sarcina*, however, the most efficient producers are the Gram-negative, acetic acid bacteria *Acetobacter xylinum* (reclassified as *Gluconacetobacter xylinus*) (BIELECKI et al., 2005).

To fine-tune the properties of BC materials, scientists have focused either on developing improved composite materials by blending with other polymers, or on manipulation of biosynthetic pathways to produce BC copolymers or miscible blends (BROWN & LABORIE, 2007).

**Figure 12.** Structure of alcohol units and of the lignin polymer (http://en.wikipedia.org/wiki/Lignin).
1.5.3 Cellulosic materials of Indian origin used in this thesis

Rice husk, mango tree and sal tree sawdust are widely available agri-forest lignocellulosic wastes that could be exploited for various value-adding applications such the development of cellulose-based materials, biosorbents for waste water treatment and industrial waste streams (e.g. removal of heavy metals and textile dyes), as well as solid carriers for cell immobilization for use in various bioprocesses and natural biofilters for cold pasteurization of liquid foods, as proposed in this thesis (KUMAR et al., 2014). India produces over 25 million tons of rice husk every year that are mainly used as a fuel in small and large scale industries for electrical power generation and thermal needs (SUN & GONG, 2001).

1.5.3.1 Mangifera indica (mango tree)

*Mangifera indica* commonly known as mango tree is a large evergreen tree belonging to the family *Anacardiaceae* Table 5. It is a cultivated plant and the fruit is used as food for human consumption. Mango also has nutritional value as fodder for livestock. It can be used as a standing feed reserve so that livestock can survive the critical period of feed scarcity during the dry season (ISAH et al., 1999).

The tree grows 10-45 m in height, with foliage of dome-shaped dense, heavily branched, with stout trunk. The leaves are mostly spiral and arranged on branches, linearly oblong, lanceolate– elliptical, pointed at both ends. The leaf blades are mostly about 25 cm long and 8 cm wide, reddish and thinly flaccid when first formed and release an aromatic odor when crushed. The bark of the tree is rough brown in color. Flowering panicles consist of about 3000 tiny whitish red or yellowish green flowers. Fruit are large drupes varying in shape and size, and generally have thick yellow pulp, single seed, and thick yellowish-red skin when ripe (Figure 13).

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<td>Class</td>
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<td>Order</td>
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<td>Family</td>
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<td>Genus</td>
<td>Mangifera</td>
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<td>Species</td>
<td><em>M. indica</em> L.</td>
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Mango wood is a natural and renewable resource that can be used as an alternative to fossil resources such as petroleum. Mango nanocellulose has been proposed for the development of novel polymeric materials as material suitable to reinforce various nanocomposites. The method proposed by HENRIQUE et al., (2013) for nanocellulose extraction involved acid hydrolysis of ground delignified mango seeds at 40°C for 10 min, with 20 mL of 11.21 M H₂SO₄ per gram of cellulose. Mango nanocellulose crystals were needle-shaped (average medium length ~123 nm, and diameter ~ 4.59 nm), with high crystallinity (90.6%), and good thermal stability (around 248°C). The sawdust from the mango wood has been utilized in various bioprocesses, such as for biosorption of heavy metals (HUBEE et al., 2011).

### 1.5.3.2 Sorea robusta (sal tree)

*Sorea robusta* Gaertn tree (*Table 6*) are large sub deciduous trees that grow in a broad range of well drained soils (*Figure 14*). Due to the low ground water table and porous soils in the region the soil surface is rarely inundated during the monsoon period (TIMILSINA et al., 2007). In India, the species is dominantly distributed on the plains and lower foothills of the Himalayas and also along the valleys of north towards a stretch of Nepal (GAUTAM, 1990).

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<td>Genus</td>
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<td>Species</td>
<td><em>S. robusta</em> Gaertn</td>
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The forests are distributed dominantly on alluvium along with that on ancient, crystalline rocks. The tree is moderate to slow growing and can attain heights of 30–50 m with 3–3.5 m diameter. Sal trees are evergreen or semi evergreen, rarely deciduous in dry season. The xylem contains aromatic resin in intercellular resin canals. The bark of the young tree is smooth, with a few long deep and vertical furrows. The sapwood is thick and whitish in color and is less durable, where as the heartwood becomes dark brown to black in color on exposure to external environment. The wood pores are mostly filled with resin. The leaves are large leathery ovate-oblong in shape. The flowers are whitish-yellowish in color, depending on the species, and come in auxiliary racemose panicles covered with white pubescence. Young trees have a linear crown, which becomes rounder and flatter with aging.

Sal is widely used in Indian ethnomedicine (Ayurveda and Unani medicine) for diverse ailments. In indigenous system the plant resin or oleo resin (gum) is used to treat diarrhea, dysentery and gonorrhea (MUKHERJEE et al., 2013). The use of sal tree wood cellulose for biotechnology or nanotechnology applications has not been reported. Porous delignified sal sawdust has been proposed (in the frame of this thesis) as cell immobilization carrier and promoter of fermentation processes (KUMAR et al., 2014).

1.5.3.4 *Oryza sativa* (rice husk)

Rice (*Oryza sativa* L.) is the world's third largest crop, behind maize (corn) and wheat. It's a dietary staple for a large part of the world’s human population, making it the most consumed cereal grain. The botanical classification of *O. sativa* L. is shown in Table 7.
Table 7. Botanical classification of *Oryza sativa* L.

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<th>Kingdom</th>
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<td>Genus</td>
<td>Oryza</td>
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<td>Species</td>
<td><em>Oryza sativa</em> L.</td>
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Figure 15. Leaf, flower and full grown *O. sativa* L.

There are two species of rice in general (*O. sativa* and *O. glaberrima*), native to tropical and subtropical southern & southeastern Asia and to Africa. It’s an annual plant, growing up to 1-1.8 m tall or more depending on the rise of water level. It has long slender leaves 50-100 cm long and 2-2.5 cm broad. Each node of the culm bears a leaf consisting of leaf sheath, leaf blade, auricles, ligules, flag leaf, panicle-spikelet, lemma-palea, and inflorescence. The small wind-pollinated flowers are produced in a branched arching to pendulous inflorescence of 30-50 cm long (Figure 15). The seed is a grain (caryopsis) 5-12 mm long and 2-3 mm thick. The seeds of the rice plant are first milled using a rice huller to remove chaff (the outer husks of the grain). This process may be continued, removing the germ and the rest of the husk, called bran to finally produce white rice.

1.5.4 Applications of delignified cellulosic materials

As discussed above, the interest in delignified cellulose, especially microfibrillated and nanocrystalline cellulose, has been increasing exponentially. During the last decade, this biomaterial was essentially been used as promoter and cell immobilization carrier in fermentations and in polymer technology for making reinforced biocomposites. Its nano/micro-scale dimensions and its microporous tubular structure have encouraged the emergence of new high-value applications.
previous years, its mode of production has completely changed, as many forms of optimization have been developed. New sources, new mechanical processes, and new pre- and post-treatments are currently under development to reduce the high energy consumption and produce new types of cellulosic materials on an industrial scale. The nanoscale characterization possibilities of different delignified cellulose are thus increasing intensively.

Cellulosic materials are very popular as immobilization carriers for alcoholic beverage production, due to their food grade purity, low cost and availability all year round. Delignified cellulosic materials have been successfully used as carriers for the development of immobilized cell biocatalysts for use in various bioprocesses related to food and fuel industries such as alcoholic and lactic acid fermentations for alcoholic beverages and dairy products production (KOUTINAS et al., 2012; BEKATOROU et al., 2014), as described in more detail in Chapter 6.

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**Chapter 6 - Food fermentations**

**1.6.1 Alcoholic fermentation**

Alcoholic fermentation takes place, through the glycolytic (Embden-Meyerhof-Parnas) pathway, i.e. the metabolism of hexose sugars into pyruvate. Under anaerobic conditions and high initial substrate concentration, pyruvate is decarboxylated by pyruvate decarboxylase and thiamine pyrophosphate as cofactor, into acetaldehyde and \( \text{CO}_2 \). Acetaldehyde acts as an electron acceptor oxidizing NADH with the formation of ethanol in order to regenerate NAD+ and allow ATP synthesis to proceed under these conditions (Figure 16). Theoretically, 1 g of sugar should yield 0.51 g of ethanol and 0.49 g \( \text{CO}_2 \). However, the usual yield of alcoholic fermentation is about 0.46 g ethanol and 0.44 g \( \text{CO}_2 \), due to heat losses and other yeast metabolic activities (BEKATOROU, 2015).

**1.6.1.1 Microbiology**

The yeast *Saccharomyces cerevisiae* is the most widely used species for alcohol production because it is remarkably tolerant to high concentrations of sugar, alcohol, \( \text{SO}_2 \), low pH, low temperatures and high pressures. It can completely utilize the sugars during beer or wine fermentations, producing low amounts of undesirable compounds such as hydrogen sulfide, acetic acid, and urea. Because of its low respiratory potential, it converts sugars mainly to alcohol and flavor desirable compounds rather than microbial biomass in the absence of oxygen (JACKSON, 1994; BEKATOROU, 2015). Different strains can be selected depending on the desired characteristics of the final products, and are commercially available to cover the needs of the alcoholic fermentation industries, including brewer’s, wine and
distiller’s yeasts. Spontaneous fermentations (e.g. traditional wine making), involve various yeast species, which may dominate at different stages of the process. Many other species have been reported to be alcohol producers, such as several fungi (Aspergillus, Fusarium and Mucor) and bacteria (Zymomonas mobilis).

The basic nutritional requirements for yeast growth are water, nitrogen and carbon sources, oxygen, phosphorus, magnesium, trace minerals, and vitamins. The principal factors that affect alcoholic fermentation processes are mainly the availability and type of carbon and nitrogen sources, temperature and pH, oxygen and alcohol levels, and minor substances that affect yeast metabolism such as minerals, vitamins, etc. (JACKSON, 1994; BEKATOROU, 2015).

![Figure 16. Overview of the alcoholic fermentation (BEKATOROU, 2015).](image-url)

**1.6.1.2 By-products**

A variety of minor volatile and non-volatile by-products may be formed during alcoholic fermentation as a result of yeast biochemical pathways (e.g. amino acid metabolism), chemical reactions between raw material constituents (e.g. esterification reactions), and yeast conversion of non-sugar compounds (e.g. diacetyl reduction). The concentration of volatile by-products (congeners) in the final product play a crucial role in its flavor and overall quality, and depends on the used strain, temperature, pH and the composition of the raw material (JACKSON, 1994; BEKATOROU, 2015). The most important volatile by-products of alcoholic fermentation are listed below:

**Methanol**

Methanol is produced by the enzymatic hydrolysis of methyl esterified carboxyl groups of polygalacturonates (pectins) by pectin methyl esterase enzymes (PME). It not an alcoholic fermentation by-product but usually occurs due to contact of raw material pectin substances with PMEs during alcoholic fermentation processes (e.g.
after crushing of grapes for wine making). Methanol is considered a toxic compound that can cause blindness in humans when consumed in concentrations higher than 500 mg/L but is generally detected at low levels in non-adulterated consumable alcoholic beverages (BEKATOROU, 2015).

**Esters**

Over 100 different esters have been identified in alcoholic beverages. Esters are considered important aroma components of fermented drinks due to their fruity/flowery aromas and low odor threshold values. They are produced during fermentation as by-products of yeast lipid metabolism (alcoholsysis of acylCoA compounds), or by slow esterification reactions between alcohols and fatty acids during aging. Important esters in fermented beverages are ethyl acetate (fruity below 150 mg/L), isoamyl acetate (banana), benzyl acetate (apple), and ethyl hexanoate (apple, aniseed), ethyl octanoate (fruity, fat) and ethyl decanoate (brandy-like) (JACKSON, 1994; BEKATOROU, 2015).

**Carbonyl compounds**

Most aldehydes found in alcoholic beverages are produced during fermentation (e.g. acetaldehyde), processing (e.g. furfural), or extracted from oak cooperage (e.g. cinnamaldehyde and vanillin). Acetaldehyde has a flavor threshold of 10–25 mg/L and is usually considered off-odor above these values (herbaceous). Important ketones derived from the raw material are mainly the norisoprenoid ketones, β-damascenone (exotic flower or rose), and α-ionone. Others are produced during fermentation and usually have no sensory significance, with the major exception being diacetyl (2,3-butanedione). Diacetyl imparts buttery, nutty, or toasty flavors at low levels (<5 mg/L) and is a key aroma compound in beer, where it is slowly converted by yeast to odorless derivatives. These reactions take place during maturation at very low temperatures (~0 °C), in a time and energy consuming process known as diacetyl rest (JACKSON, 1994; BEKATOROU, 2015).

**Higher alcohols**

Higher (or fusel) alcohols (more than two carbon atoms) are mainly produced by yeast during fermentation as a result of amino acid metabolism. Few exceptions such as hexanols, benzyl alcohol, and 2-phenylethanol, are derived from the raw material. The most abundant alcohols are 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol. They contribute more positively to aroma complexity at low concentrations (<300 mg/L). At higher concentrations they may musk the product aroma with their strong pungent smells (JACKSON, 1994; BEKATOROU, 2015).

**Organic acids**

Acidity in alcoholic beverages is commonly classified into two categories – volatile and fixed, referring to acids that can be removed by steam distillation or poorly volatile acids, respectively. Acetic acid is the major volatile acid found in wines, but
other carboxylic acids such as formic, butyric, and propionic, as well as longer chain fatty acids also may be involved. All volatile acids have specific odors but usually occur at detectable levels only as a result of microbial spoilage (JACKSON, 1994; BEKATOROU, 2015).

1.6.2 Lactic acid fermentation

Lactic acid fermentation is a type of fermentation where sugars are metabolized following the glucoylytic pathway resulting in the production of lactic acid either as the unique product (homofermentative metabolism) (Figure 17) or along with ethanol and CO₂ (heterofermentative metabolism). Under conditions of excess glucose and limited oxygen, homolactic lactic acid bacteria (LAB) catabolize one mole of glucose to yield two moles of pyruvate. Intracellular redox balance is maintained through the NAD+ regeneration by pyruvate reduction to lactic acid. This process yields two moles of ATP per glucose consumed as in the case of alcoholic fermentation (BOSNEA, 2009; TOLDAR, 2008).

![Figure 17. Overview of homolactic fermentation in lactic acid bacteria (LAB).]

1.6.2.1 Microbiology

Microorganisms that can produce lactic acid can be divided into two groups: bacteria and fungi. Although most investigations of lactic acid production were carried out using LAB, filamentous fungi, such as Rhizopus (e.g. R. oryzae and R. arrhizus) can utilize glucose aerobically to produce lactic acid. Rhizopus species have amylolytic enzyme activity, which enables them to convert starch directly to L(+)lactic acid. Therefore, fungal fermentation is advantageous because it requires no substrate pretreatment and produces pure lactic acid isomer; however, it requires vigorous aeration because such fungi are obligate aerobes, and has low production rates due
to mass transfer limitations and formation of by-products, such as fumaric acid and ethanol (WEE et al., 2006).

Based on the fermentation pattern, there are two broad metabolic categories of LAB, i.e. homofermentative and heterofermentative species. The first category includes some Lactobacilli and most species of Enterococci, Lactococci, Pediococci, Streptococci, Tetragenococci, and Vagococci. The second category includes Leuconostocs, some Lactobacilli, Oenococci, and Weissella species. The homofermentative LAB usually metabolize glucose via the glycolysis pathway resulting only in lactic acid as a major end-product, and are the only available species for commercial production of lactic acid (WEE et al., 2006).

Most of the LAB used belong to the genus Lactobacillus, and specifically the species L. delbrueckii. The genus Lactobacillus belongs to the phylum Firmicutes, class Bacilli, order II Lactobacillales, and family Lactobacillaceae. Lactobacilli are Gram-positive, catalase negative, non-spore-forming, rod-shaped bacteria. They have complex nutritional requirements, including carbohydrates, fatty acids or fatty acid esters, salts, nucleic acid derivatives, and vitamins (DE ANGELIS & GOBBETTI, 2011). In the past, there has been some confusion in the naming of thermophilic lactobacilli with often mistaken identification particularly between L. delbrueckii subsp. bulgaricus and L. helveticus. These two species can be clearly separated by genetic criteria, but for practical purposes as dairy starters some strains from the two species are similar (SHARPE et al., 1957).

![Diagram of Major Pathways of Hexose Fermentation in LAB](LIU, 2003)

**Figure 18.** Major pathways of hexose fermentation in LAB (LIU, 2003).
**L. delbrueckii** subsp. **bulgaricus** is a homofermentative lactic acid bacterium that can provide a continuous bioprocess with high volumetric productivity and optically high purity of D(-)-lactic acid under anaerobic conditions (LIU, 2003).

### 1.6.2.2 By-products

*Lactobacilli* as starters may play different roles in the metabolism of lactose, lactate, and citrate, and in proteolysis and lipolysis, which are considered the primary events for characterizing product flavor, such as during cheese ripening. Nevertheless, in many dairy fermentations, whether they contribute significantly to the flavor or not, it will depend on many other factors (e.g. product type and composition, strain type and density, storage temperature and time) (DE ANGELIS & GOBBETTI, 2011).

### 1.6.2.3 Production and uses of lactic acid

![Potential routes of lactate formation from sugars, polyols, organic acids and amino acids in LAB (LIU, 2003).](image)

Lactic acid is one of the most widely used natural organic acids in pharmaceuticals, chemicals, food industries, environment friendly green solvents, and biodegradable plastics (PAULI & FITZPATRICK, 2002). It can either be produced by chemical synthesis or by microbial fermentation. Chemical synthesis from petrochemical resources always results in racemic mixture of DL-lactic acid, which is the major disadvantage of this approach (HOFVENDAHL & HAHN-HAGERDAL, 2000). Conversely, microbial lactic acid fermentation offers an advantage in terms of the utilization of renewable carbohydrate biomass, low production temperature, low
energy consumption, and the production of optically high pure lactic acid by selecting an appropriate microbial strain (ILMEN et al., 2007; PANDEY et al., 2010). Presently, almost all lactic acid produced globally is manufactured by fermentation routes. However, the widely used choice substrates for lactic acid fermentation are expensive refined sugars necessitating the investigation of various cheap and renewable substrates like agro-industrial by-products and wastes (ALTAF et al., 2005).

Traditional production of lactic acids typically uses starch derived from food crops, but this process may affect the global food supply. Waste lignocellulosic biomasses can be used to produce lactic acid, but require the breakdown of cellulose into fermentable sugars, by either acid or enzymatic hydrolysis. The enzymatic hydrolysis can be done under mild conditions avoiding the use of toxic and corrosive chemicals. Hydrolysis and fermentation can be performed simultaneously offering better yields by decreasing end-product inhibition (WEE et al., 2006; ZHANG & VADLANI, 2013).

1.6.2.4 Cheese way fermentation

Whey is the liquid waste from the dairy industry, which is produced in large quantities annually and has a high organic load making its disposal both a wasteful practice and a significant input of biological oxygen demand into the environment. On the other hand, whey has an appreciable nutritional value as it contains significant amounts of proteins, lactose, organic acids, fat, vitamins and minerals, and its conversion into products of added value is important from both economical and environmental points of view. The composition of whey (e.g. high salinity) and high temperature at the moment of its production in the dairy industry does not allow easy microbial conversion (KOUTINAS et al., 2008). Recent advances to deal with this problem include the use of selected cultures (e.g. mixed cultures or thermotolerant strains) or modified microorganisms (e.g. those expressing \( \beta \)-galactosidase activity) and optimized processes to produce or recover foods or chemicals of added value, such as single cell protein (SCP), organic acids, enzymes, lactose and ethanol. The majority of efforts for whey utilization employ LAB for production of lactic acid and polysaccharides and to a lesser extent, production of SCP, probiotics and other applications. However, the complete substrate utilization and good biomass yields are highly dependent on process conditions (aeration, agitation, \( \text{pH} \), temperature), nutrient composition and the genetic characteristics of the strain used (KOUTINAS et al., 2008).

1.6.3 Fermentation processes advantaged by delignified cellulosic materials

Alcoholic fermentation using cell immobilization techniques has far been proved to be very efficient in past three decades, due to its technical and economical advantages over those of free cells systems. Specifically, the advantages of immobilized cells for alcoholic beverages production include: (i) achievement of high cell densities in the bioreactors, higher productivities and shorter process times; (ii)
1. Introduction

protection against shear forces and stress leading to extended operational stability; (iii) feasibility of continuous processing, easy product recovery, and reusability of the biocatalyst; (iv) feasibility of low-temperature fermentation, which can lead to improved product quality; (v) reduction of maturation times; (vi) reduced contamination risk due to the higher cell densities and increased fermentation activity; (vii) reduction of investment and energy costs due to small installations, less separation and filtration requirements, and higher productivities (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014).

Delignified cellulosic materials have been widely studied as immobilization supports for alcoholic fermentation processes leading to products with improved quality and higher productivities (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014). The delignification treatment yields cellulosic materials with more porous structures at the micro and nanoscales. These structures favor the entrapment of yeast or bacterial cells and seem to also affect the rates of fermentation by attraction of sugars on the cellulose surface by hydrogen bonding, thus providing a continuous flow of sugar towards the immobilized cells (KOUTINAS et al., 2012; GANATSIOS et al., 2014).

Specifically, advantages in maltose and glucose fermentation at extremely low temperatures (5-10°C) using the alcohol resistant and cryotolerant yeast strain S. cerevisiae AXAZ-1 (also utilized in this thesis) immobilized on porous delignified wood sawdust (or tubular cellulose, TC) have been reported (KOUTINAS et al., 2012; GANATSIOS et al., 2014). Pure maltose and glucose media were examined as model substrates to evaluate the potential effect of TC on the rate of fermentation of substrates containing these sugars. The use of TC sharply accelerated the fermentation rates compared to free cells (FC) in suspension, and allowed fermentation to proceed even at extremely low temperature (0-5°C). From the results of maltose and glucose fermentations at various temperatures, it was concluded that the effect of TC was higher at lower temperature and that its promotional effect on fermentation rate had to be at the step of sugar uptake. Specifically, it is suggested that the presence of TC increased sugar uptake rate by the immobilized cells due to attraction by hydrogen bonding on the TC surface and continuous pumping of maltose towards the cells. Calculation of the activation energy of maltose or glucose fermentations at 5, 10 and 15°C showed that it was reduced by more than 30% when cells immobilized on TC were used (KOUTINAS et al., 2012; GANATSIOS et al., 2014).
The surface structure of TC has been studied by scanning electron microscopy, porosimetry, and X-ray powder Diffractometry (XRD) (KOUTINAS et al., 2012). The cumulative surface area of the TC pores was found to be 0.8-0.89 m$^2$ g$^{-1}$ as indicated by porosimetry analysis. This surface is relatively small compared with other porous materials such as γ-alumina, however, using a natural organic material is attractive from the point of view that it is safer for bioprocess applications and is better accepted by consumers.

Similar advantages have been reported for the use of TC in malolactic (MLF) and lactic acid fermentation processes. MLF (conversion of malic acid to lactic acid by certain types of bacteria) may occur in bottled wines that have not been adequately preserved with SO$_2$, causing undesirable turbidity and development of off-flavors. The use of selected immobilized lactic acid bacteria was found suitable to accelerate controlled MLF by higher cell densities and increased tolerance to inhibitory wine constituents, leading to flavor improvements. For example the use of immobilized $O$. oeni on TC led to improvements of MLF in wine making, and this effort was further enhanced by the development of a two-layer composite biocatalyst for simultaneous alcoholic and MLF (BEKATOROU et al., 2014). The biocatalyst consisted of TC with entrapped $O$. oeni cells, covered with starch gel containing $S$. cerevisiae AXAZ-1. The significance of such composite biocatalysts is the feasibility of two or three bioprocesses in the same bioreactor, thus reducing production cost in the food industry.

Kefir immobilized on TC was also found suitable for continuous whey fermentation supplemented with 1% raisin extract or molasses. The fermented whey could be exploited as raw material to produce kefir-like whey-based drinks, food-grade and fuel alcohol. However, studies on the effect of TC on lactic acid fermentation by LAB using lactose containing waste streams, such as cheese whey, are limited (KOUTINAS et al., 2009; KUMAR et al., 2014).
Finally, TC was found suitable for the development of non-fermentation applications, e.g. for cold pasteurization processes acting as a biofilter for cell removal (KOUTINAS et al., 2012; GIALELI et al., 2015).

**Chapter 7 – Immobilized cell technologies**

1.7.1 Cell immobilization

Whole cell immobilization is defined as “the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity”. Immobilization resembles the conditions in which microbial cells are found in nature, taking into account that they are usually found adhered and growing on different kinds of surfaces. The considerable research and industrial interest in the use of immobilized cells for alcoholic and other food and fuel-related fermentation applications is due to the numerous advantages that such technologies offer compared to conventional free cell systems. The techniques and materials used for microbial cells immobilization, their advantages, research efforts and practical applications have been recently reviewed (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014).

1.7.1.1 Techniques and carriers

Cell immobilization has been used in all types of alcoholic beverages production and various other biotechnological processes, and therefore many such techniques have been developed, which can be grouped into four major categories as illustrated in Figure 21 (KOURKOUTAS et al., 2004):

(a) Immobilization on a solid carrier;
(b) Immobilization by entrapment in a porous matrix;
(c) Carrier-free immobilization;
(d) Containment behind barriers.

**Cell immobilization on a solid carrier** can be done by physical adsorption due to electrostatic forces, or by covalent binding between the cell membrane and the carrier, or by growth of the cells into natural cavities on a surface, and therefore containment of the cells due to entrapment and/or a combination with electrostatic and other weak forces. In this type of immobilization, the adhered cells may grow and escape, and therefore may be in equilibrium with free cells in suspension (BEKATOROU et al., 2014). Such immobilized cell systems have been extensively used due to the ease of the immobilization technique, which in some cases can be achieved by simple contact of a cell suspension with the carrier for a small time period, as in the case of cellulosic materials used in this thesis.
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Examples of solid carriers used in this type of immobilization are cellulosic materials such as DEAE-cellulose, wood, sawdust, delignified sawdust, cereal bran, etc., and inorganic materials such as polygorskite, montmorillonite, hydromica, porous porcelain, porous glass, pumice stone, etc. Solid materials like glass or cellulose can also be treated with various chemicals (e.g. polycations, chitosan, etc.) to improve their characteristics as cell binding carriers (BEKATOROU et al., 2014).

**Entrapment of cells in a porous matrix** is a more definite type of immobilization, in which cells are either allowed to penetrate into a porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed in situ into a culture of cells. Other entrapment methods are based on inclusion of cells in a rigid network, which allows mass transfer of nutrients and metabolites. This approach for cell immobilization has been by far the most popular for research studies, including alcoholic beverages production, with main advantage that it is a simple technique that proceeds under very mild conditions and is therefore compatible with most living cells.

The most frequently used than any other materials for this application are hydrogels of natural polysaccharides such as alginates, cellulose, k-carrageenan, agar, pectic acid and chitosan, or other polymeric matrixes such as polyacrylamide, gelatin, collagen and polyvinyl alcohol (BEKATOROU et al., 2014).
1. Introduction

Carrier-free immobilization can be done by self-aggregation by natural flocculation or artificially induced using cross-linking agents. Cell flocculation has been defined as an aggregation of cells to form a larger unit or the property of cells in suspensions to adhere in clumps and sediment rapidly. Flocculation can be considered an immobilization technique since the large size of the aggregates facilitates use in bioreactors, such as packed-bed, fluidized-bed and continuous stirred-tank reactors. It is the most simple and inexpensive immobilization method, although it is very difficult to predict and control (BEKATOROU et al., 2014).

Containment of cells behind a barrier can be achieved either by the use of semipermeable membranes, to isolate the cells from the bulk liquid, or by entrapment of cells in a microcapsule, or by cell immobilization on the interaction
surface of two immiscible liquids. This type of immobilization is ideal when cell free product and minimum transfer of compounds are required, and it is widely used in cell recycling and continuous processes. The major disadvantages of membrane immobilization techniques are mass transfer limitations (supply of oxygen and nutrients to the cells and the removal of carbon dioxide), and possible membrane plugging caused by cell growth (BEKATOROU et al., 2014).

1.7.1.2 Effects of immobilization on microbial cells

Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization, such as effects on the energetic metabolism activation, targeted protein expression to support the altered metabolic behavior of immobilized cells, altered growth rates, increased substrate uptake and product yield, increase in storage polysaccharides, altered yield of fermentation by-products including flavor compounds, higher intracellular pH values, increased tolerance against toxic and inhibitory compounds, increased hydrolytic enzyme activities, modifications in the nucleic acid contents, etc. Parameters that have been considered responsible for these alterations include mass transfer limitations, disturbances in the growth pattern, surface tension and osmotic pressure effects, reduced water activity, cell-to-cell communication, changes in the cell morphology, altered membrane permeability, etc. (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014).

Due to the above reasons, immobilized cells showed enhanced viability and stability during freezing, freeze-drying and thermal drying, which was exploited for the development of ready-to-use dried biocatalysts for commercial applications. For example, research on the development of active dried immobilized yeast on delignified cellulosic materials was carried out, mainly aiming to optimize freeze-drying or simpler, mild, and cost effective thermal drying techniques. The immobilized biocatalysts retained their viability during storage and showed high productivity and stability for glucose, wine and beer fermentations, leading to products of similar quality to those produced by fresh immobilized cells and of improved quality compared to free cells (BEKATOROU et al., 2014; TSAOUSI et al., 2011).

1.7.2 Immobilization techniques and materials used in this thesis

The materials proposed in this thesis as cell immobilization carriers to promote alcoholic and lactic acid fermentation are wood sawdust and rice husk of Indian origin as described before. The materials were analyzed (proximate analysis and surface/crystallinity characteristics) and were used after alkaline delignification. The delignified materials (TCs) were successfully used as immobilization carriers to promote alcohol and lactic acid production as discussed in the Result & Discussion section (KUMAR et al., 2014).

To evaluate the effect on porosity and crystallinity characteristics, composite materials by blending with various biopolymers (PLA, PHB, Ca-alginate) were also prepared and were evaluated as the plain TCs. Apart from the modified surface
properties, composite materials containing different microorganisms, co-immobilized, are useful to simultaneously conduct different bioprocesses into the same bioreactor (e.g. malolactic fermentations wine). This could lead to reduction of both production and investment costs. Separate entrapment of cells in the different matrices to form a composite multispecies biocatalyst could also help avoid species inhibition problems (SERVETAS et al., 2013).

They TCs were also carbonized in order to evaluate the effect of this treatment on their surface/porosity characteristics and the suitability for use in bioprocessing.

Apart from the development of food fermentation processes, the above materials are also proposed as biofilters for cold pasteurization of liquid foods as discussed below in Chapter 8.

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**Chapter 8 – Advances in food pasteurization**

### 1.8.1 Thermal pasteurization

In 1824 WILLIAM DEWEES suggested that milk for the infants be pre-heated near to the boiling (but not boiled) then cooled simultaneously as preparation for infants feeding. The major credit for the invention of a mild method of processing foods now particularly concerned to milk and other liquid foods has been given to Louis Pasteur, after whom the term pasteurization was named. Pasteurization is a thermal process applied to a product aiming to avoid public health hazards arising from the presence of pathogenic (and spoilage) microorganisms. It has become a widely accepted effective means of destroying vegetative pathogens in food products, with the least possible damage to the sensory qualities of the product. Pasteurization is applied mainly in relatively sensitive products such as fruit juices and milk, where more severe sterilization processes (as in canning) cannot be applied as they would destroy the product texture and nutritional value. The heat treatment also reduces the general microbial populations, so that an increased shelf-life is normally obtained (WILBEY, 2003).

Thermal pasteurization is one of the traditional physical processes, still commonly being used today for the decontamination of food as an efficient, environmentally friendly, preservative-free and inexpensive means of food processing when compared with other preservation technologies. Heat as a thermal energy inactivates pathogens and helps in developing typical flavors, aromas, texture and color of a cooked food. Pasteurization in this concern is a process that results in safer foods with longer shelf-life, since mild temperatures are applied for a specified time (FILIPA et al., 2012). The mild temperatures applied to foods (<95°C) for a specified time allow greater retention of the original properties of the raw food while inactivating vegetative pathogens such as *Salmonella* and other respective microorganisms. Plate heat exchangers and tunnel pasteurizers are most commonly
used as a continuous thermal pasteurization of liquid foods such as juices and milk (MOYER & AITKEN, 1980).

Table 8. Pasteurization conditions used for milk products (http://www.milkfacts.info)

<table>
<thead>
<tr>
<th>Pasteurization Type</th>
<th>Typical Product</th>
<th>Temperature (°C)</th>
<th>Holding Time</th>
<th>Typical Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batch, vat</strong></td>
<td>Milk</td>
<td>62.8</td>
<td>30 min</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td>Viscous products/products with &gt;10% fat/products with sweeteners</td>
<td>65.6</td>
<td>30 min</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td>Dessert mixes</td>
<td>68.3</td>
<td>30 min</td>
<td>Refrigerated</td>
</tr>
<tr>
<td><strong>Continuous, high temperature short time (HTST)</strong></td>
<td>Milk</td>
<td>71.7</td>
<td>15 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td>Viscous products/products with &gt;10% fat/products with sweeteners</td>
<td>74.4</td>
<td>15 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td>Egg nog, frozen dessert mixes</td>
<td>79.4</td>
<td>25 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82.2</td>
<td>15 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td><strong>Continuous, higher heat shorter time (HHST)</strong></td>
<td>Milk</td>
<td>88.3</td>
<td>1 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>0.5 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.8</td>
<td>0.1 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96.2</td>
<td>0.05 sec</td>
<td>Refrigerated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.01 sec</td>
<td>Refrigerated, extended storage</td>
</tr>
<tr>
<td><strong>Continuous, Ultrapasteurization</strong></td>
<td>Milk and cream</td>
<td>137.8</td>
<td>2 sec</td>
<td>Refrigerated, extended storage</td>
</tr>
<tr>
<td><strong>Aseptic, ultra high temperature (UHT)</strong></td>
<td>Milk</td>
<td>135-150</td>
<td>4-15 sec</td>
<td>Room temperature</td>
</tr>
<tr>
<td><strong>Sterilization</strong></td>
<td>Canned products</td>
<td>115.6</td>
<td>20 min</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

Being a mild thermal treatment, possible microbial survivors (e.g. spore formers) might be present in the food after the pasteurization process. For reasons of public safety, low-acid (pH 4.6) raw and pasteurized foods, such as foods from animal origin, are generally stored, transported and sold under refrigerated conditions (below 7°C) and with a limited shelf-life, to minimize the outgrowth of pathogenic microorganisms until consumption. In the case of sterilized foods, all microorganisms including spore formers are inactivated and the product can be safely stored at room temperature (SILVA & GIBBS, 2009).

In principle, only thermoduric bacteria survive pasteurization. Usually, the bacterial populations in milk, expressed as mesophilic aerobic counts or standard plate counts, is reduced by at least 90% by pasteurization. Besides pathogens in their vegetative form, all coliforms and most, if not all, psychrotrophs are destroyed (BURTON, 1986).
Among LAB, heat sensitivity varies according to species (TURNER et al., 1986). Thermophilic LAB are more heat resistant.

One major disadvantage of pasteurization processes for the food and drug industries is their high operational cost and the reduction of various nutritional qualities of food products. Despite of the cost and nutrition related disadvantages pasteurization is extensively used in food processing. Alternative technologies such as membrane filtration are also used to remove microorganisms like Leuconostoc oenos cells from wine; however these techniques are very expensive and difficult to process at industrial level due to fast membrane fouling (KOUTINAS et al., 2012). Thus cold pasteurization of liquid foods in this concern using cheap filters based on natural cellullosic materials is an interesting perspective.

1.8.1.1 Effects on food quality

During storage, liquid foods undergo an important number of deterioration reactions such as ascorbic acid degradation, cloud loss, microbial spoilage, development of off-flavor, changes in color, texture, and appearance, and an important quality loss. Thermal processing inactivates microorganisms and enzymes that cause spoiling effectively but can also reduce the organoleptic and nutritional quality (ZULUETA et al., 2013; QIN et al., 1995). Time/temperature protocols of pasteurization processes have been optimized to minimize the effects caused from the exposure of some foods to heat. However, optimization of pasteurization treatment on time/temperature factors is still a major challenge for the incipient processing industry of some products (VEGARA et al., 2013).

Heating in products such as fruit juices just after the pressing inhibits native polyphenol oxidase (PPO) enzymes that cause oxidative browning (SKREDE & DURST, 2000), but can cause irreversible losses of nutritional compounds, undesirable changes in physicochemical properties and alteration of their antioxidant properties (ZULUETA et al., 2013). The presence of furfural and 5-hidroxymethylfurfural (HMF) in stored citric products is an indicator of their quality loss since these compounds are indicators of excess of temperature and storage time.

Regarding milk, although the public health benefits of pasteurization are well established, advocate organizations continue to promote raw milk as "nature’s perfect food." These groups claim that pasteurization destroys important vitamins and that raw milk consumption can prevent and treat allergies, cancer, and lactose intolerance. A systematic review and meta-analysis was completed to summarize available evidence for these claims. Qualitatively, vitamins B12 and E decreased following pasteurization, and vitamin A increased. Random effects meta-analysis revealed no significant effect of pasteurization on vitamin B6 concentrations but a decrease in concentrations of vitamins B1, B2, C, and folate. It was concluded that the effect of pasteurization on the nutritional value of milk was minimal because many of these vitamins are naturally found in low levels. However, milk is an important dietary source of vitamin B2, and the impact of heat treatment should be further considered (MACDONALD et al., 2007).
1.8.2 Cold pasteurization processes

Alternative non-thermal technologies have been proposed for obtaining food products with a fresh-like appearance whilst preserving their nutritional content, such as high-pressure (CO₂ or N₂O), pulsed electric field, irradiation, microfiltration, ultrasound, bactofugation, etc. (ZULUETA et al. 2013; LESTER & ERIC, 1996; WALKLING-RIBEIRO et al., 2011; SPILIMBERGO, 2011; RIBEIRO et al., 2010).

![Figure 22. SEM micrograph of L. casei flocculation on TC filter during a cold pasteurization (5°C) process (scale bar corresponds to 10 µm) (KOUTINAS et al., 2012).](image)

Water disinfection as per recorded in the developing countries is very huge and rather complicated problem, especially in Asian countries such as India. There are several methods used for disinfection of water in India, but chlorination is the most economical and feasible. However, chlorination generates various chlorine byproducts, which are potential carcinogens, especially halogenated organic by-products such as trihalomethane. Bank filtration, is a natural approach to extract water from rivers through wells installed in neighbouring alluvial aquifers. A decrease in the concentration of pollutants can be achieved by various processes such as filtration, biodegradation, adsorption, precipitation and redox reactions between ground water and surface water (SHARMA et al., 2014).

Cold pasteurization using the delignified cellulose materials (TC) as natural biofilters, has been previously investigated using aqueous suspensions of Lactobacillus casei and S. cerevisiae, and orange and apple juices inoculated with L. casei (Figure 22). Filtration took place in a continuous bioreactor system packed with TC at 3-4°C and efficiently removed the microbial load (GIALELI et al., 2015; KOUTINAS et al., 2012). The above findings supported the use of TC for further investigations regarding the development of simple cold pasteurization processes for water ad liquid foods, based on cheap and renewable raw materials.
Chapter 9 – Thesis Aims

Fermentation and pasteurization are highly significant processes for the food industries and it will be of great interest in the near future to employ renewable natural materials as biocatalysts to attain cost-effectiveness, sustainability, health and quality benefits and consumer acceptance. The combination of waste cellulosic biomass, biopolymers, and advanced technologies such as biotechnology and nanotechnology, will play a crucial role in this respect.

The idea behind the nano/microtubing which is exploited in this thesis, was evolved from the recent concept of utilizing delignified tubular cellulose (TC) as cell immobilization carrier and promoter of alcoholic and lactic acid food, fuel and waste treatment fermentations. Thus, cellulosic materials of Indian origin (mango and sal wood and rice husk) are proposed as abundant natural ubiquitous biopolymer resources, with good grounds for the development of multi-functional nano/micro porous materials and composites to promote fermentation processes.

Additionally, the proposed TCs were investigated as natural, low cost filters for simple and cost-effective liquid food cold pasteurization processes, with water and milk as model substrates.

The TCs materials were analyzed regarding their proximate composition and structural characteristics, and they were used (1) in plain form, (2) in composites with other biopolymers (PLA, PHB, alginates) and (3) after carbonization in order to evaluate their potential for use in the above bioprocesses.
2. Experimental Part

Chapter 1 – Materials and Methods

2.1.1 Chemicals, materials and lab equipment

- PLA and PHB (Sigma Aldrich)
- Sodium alginate (Fisher chemicals)
- Calcium chloride (Fisher chemicals)
- Skimmed and semi-skimmed commercial milk (NOYNOY)
- NaOH (1% w/v) (Penta)
- KOH (1% w/v) (Penta)
- Ringer’s solution (LAB M)
- MRS broth (LAB M)
- MRS agar (Fluka Biochemical)
- Alcoholic solution 70% v/v for sterilization
- Cylindrical glass bioreactors (0.6 m height from inlet to outlet; 0.066 cm diameter; 2.052 l volume)
- Silicon pipes (5 mm diameter)
- Conical and volumetric flasks
- Test tubes with caps
- Sterilized tips (100-1000 µm)
- Pipettes of 100-1000 µm (Transferpette-BRAND)
- Cheese cloth for filtering uses
- Nylon perforated fabric for TC filter construction
- Sterilized Petri dishes
- Centrifuging tubes
- Bunsen burners
- Thermometers

2.1.2 Instruments and devices

- Agitation/heating device (Daihan Scientific)
- Peristaltic pump (Cole Parmer Instruments Co.)
2. Experimental Part

- Autoclave
- Incubation chambers
- Centrifuge (Sigma 3K12)
- Scale of high accuracy (Exacta)
- Water bath (Elma)
- Oven (Nabertherm B170)
- Vortex device (Ika-Vibro-Fix)
- pH-meter (Hanna Instruments)
- UV-VIS spectrophotometer (Jenway, UK)
- Freeze Drying System (Freezone 4.5; Labconco)
- Controlled rate freezer (BioCool, FTS Systems)
- Scanning Electron Microscope (JEOL JSM-6300)
- Sputter coater (Balzers SCD 004)
- X-ray diffractometer (Enraf Nonius FR 590)
- Porosimeter (Micromeritics TriStar 3000)
- GC/MS (Shimadzu GC-17A/MS QP5050)
- Manual SPME holder and fibre (Supelco, Bellefonte)
- HPLC-DAD (Jasco LC-2000 Series)
- HPLC-RID (Shimadzu LC-9A)
- GC-FID (Shimadzu GC-8A)
- TG/DTA (Perkin Elmer)
- Kjeldhal Automatic Micro Sample Digestion System (Pelican equipment)
- Common household refrigerator

2.1.3 Raw materials

2.1.3.1 Cellulosic materials

Sawdust from Mango wood (*M. indica*) and Sal wood (*S. robusta*), and rice husk (*O. sativa*), were obtained from a local timber producer in the Ranchi area (Jharkhand Forest), India. All materials were sieved for uniformity through 600 μm mesh sieves, and their various chemical and structural properties were analyzed (Chapter 5). The materials were evaluated for various bioprocesses development (immobilized cell biocatalysts for fermentation processes and filters for liquid food pasteurization) after delignification as described below in Chapters 2-4. The materials were also carbonized (as described in Chapter 2) and their surface/porosity characteristics were evaluated.
2. Experimental Part

2.1.3.2 Cheese whey

Cheese whey was supplied by Chelmos S.A. dairy industry, Santameri, Achaia, Greece. It contained about 50 g lactose/L and was used for the lactic acid fermentation experiments. Before use it was sterilized at 120°C and 1.5 atm for 15 min.

2.1.3.3 Water

Dionized water was used as raw material for the cold pasteurization experiments after sterilization at 120°C and 1.5 atm for 15 min.

2.1.3.4 Skimmed and semi-skimmed milk

Commercial skimmed and semi-skimmed cow's milk (NOYNOY) were obtained from local supermarkets, and were used for the cold pasteurization experiments after to dilution with sterilized deionized water (50:50).

2.1.4 Microbial strains and culture media

2.1.4.1 Lactobacillus delbrueckii subsp. bulgaricus

The strain *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20081 (DSMZ, Germany) (*L. bulgaricus*), isolated from Bulgarian yogurt, was used for lactic acid fermentation and cold pasteurization experiments. It belongs to the group of Gram-positive thermophilic, microaerophilic rod-shaped bacteria and is an obligately homofermentative species.

It was initially inoculated and grown at 37°C in MRS agar, and cell mass was obtained by subculturing (static cultures) in 500-2000 mL of media containing 55 g/L MRS broth until enough cell mass was obtained. The culture was then harvested by centrifugation at 5000 rpm for 8-10 min to obtain a culture of ~70% moisture, which was stored at 0-5°C until further use. All media were autoclaved at 120°C for 15 min prior to use.

2.1.4.2 Saccharomyces cerevisiae

The *S. cerevisiae* AXAZ-1 strain was used, which is an alcohol-resistant and psychrotolerant yeast, isolated from the Greek vineyard (Ano Ziria, Achaia, Greece) (ARGIRIOU et al., 1996), and available at the Department of Chemistry of the University of Patras. *S. cerevisiae* AXAZ-1 was inoculated and grown at 30°C, without
aeration, in synthetic media containing (g/L):

- Yeast Extract – 4
- Glucose – 40
- Ammonium sulfate – 1
- Dihydrogen potassium phosphate – 1
- Magnesium sulfate heptahydrate – 5

The culture was harvested by centrifugation at 5000 rpm for 8-10 min and stored at 0-5°C. All media were autoclaved at 120°C for 15 min prior to use.

**Chapter 2 – Preparation of biocatalysts and experimental design**

**2.2.1 Delignification of the cellulosic materials**

Sawdusts from mango and sal tree, and rice husk were delignified according to the method of BARDI & KOUTINAS (1994). Specifically, 300 g of each material was boiled in 3 L of 1% w/v NaOH solution. Distilled water was added when the liquid level decreased due to evaporation. After 3 h of continuous boiling the liquid containing the soluble lignin was removed by filtration and the delignified sawdusts (TCs) were washed thoroughly with hot water to remove any residual lignin. The TCs were sterilized at 125°C for 15 min and stored at 0-5°C until further use (for cell immobilization or for cold pasteurization filter development). Before use, they were dried overnight at 37°C.

**2.2.2 Cell immobilization on the delignified cellulosic materials (TCs)**

The three TC materials were used as carriers for the immobilization of *S. cerevisiae* and *L. bulgaricus* cells. For *S. cerevisiae* the method described by BARDI & KOUTINAS (1994) was used. Specifically, 16 g of the harvested culture was separately mixed with 800 mL of glucose synthetic medium (12% w/v) and 100 g of TC were added. The system was allowed to ferment for 6-8 h until all sugar was utilized (density 0-0.5°Be). The fermented liquids were then decanted and the biocatalysts were washed gently with fresh glucose medium and then with sterile Ringer’s solution and were used for the alcoholic fermentation experiments.

For immobilization of *L. bulgaricus*, an amount of 5 g of each TC material was added in a conical flask containing 300 mL of MRS broth and the whole was sterilized by autoclaving at 120°C for 15 min. After cooling at 40°C, 1 g of harvested *L. bulgaricus* culture was added in each flask and the mixtures were incubated at 37°C for 48 h to allow cell immobilization by natural adsorption and growth in the TC pores. The biocatalysts were washed as above and were used for the lactic acid fermentation experiments.
2.2.3 Preparation of composite (alginate/TC) immobilized cell biocatalysts

For the preparation of the alginate/TC composites, the mango, sal, and rice husk TCs with immobilized *L. bulgaricus* and 0.2 g of polylactic acid (PLA) were mixed in 50 mL of a 2% w/v Na-alginate solution. The mixture was then added drop-wise in 50 mL of a CaCl$_2$ solution (1% w/v) to obtain solidified composite beads. The composite biocatalysts were used for lactic acid fermentation experiments.

2.2.4 Preparation of TC filters for liquid food cold pasteurization

The mango, sal, and rice husk TCs were used to prepare a packed cylindrical filter to be evaluated for cold pasteurization of liquid foods (water and milk), i.e. removal of microbial load under refrigeration. The filter was prepared by packing suitable amounts of the different TCs in a thin, perforated nylon fabric to produce cylindrical filters of different lengths (35 cm/1348 cm$^3$ and 45 cm/1733 cm$^3$), which were suitable for a cylindrical bioreactor of 7 cm internal diameter (*Figure 23*).

2.2.5 Preparation of carbonized TC materials

Carbonization of the mango, sal, and rice husk TCs was done in order to increase the number of tubes and specific surface area. Dry samples of the TCs were placed in an oven (Nabertherm B170) and were treated at the temperatures of 250°C and 350°C under nitrogen flow (0.6 L/min, 1.5 bar) for 6 and 12 h, respectively.

2.4.6 Experimental design

The above biocatalysts were evaluated for their efficiency in lactic acid fermentation, alcoholic fermentation and liquid food pasteurization processes, as synopsized in the Table 9 below and described in more detail below, in Chapter 3.
2. Experimental Part

Table 9. Experimental design

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Process</th>
<th>Cold pasteurization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactic acid fermentation;</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. bulgaricus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcoholic fermentation;</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. cerevisiae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Whey</td>
<td>Skim milk</td>
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<tr>
<td></td>
<td>Lactose</td>
<td>Semi-skimmed milk</td>
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<tr>
<td></td>
<td>Glucose</td>
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<td></td>
<td>Wine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>Mango TC/alginate</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Rice TC/alginate</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sal TC/alginate</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Mango TC</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Rice TC</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sal TC</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Free cells</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Chapter 3 – Fermentations using the TC biocatalysts

2.3.1 Lactic acid fermentation of lactose using TC/*L. bulgaricus* biocatalysts

Lactic acid fermentation was carried out at 37°C using 5 g of each the immobilized biocatalysts (mango, sal and rice husk TCs with immobilized *L. bulgaricus* cells) in 300 mL of lactose synthetic media containing (g/L):

- Lactose – 20
- Yeast extract – 5
- Dihydrogen potassium phosphate – 2
- Sodium acetate trihydrate – 5
- Triammonium citrate – 2
- Manganese sulfate – 0.05
- Magnesium sulfate – 0.02

The fermented products were analyzed for lactic acid concentration and residual sugar by HPLC, and volatile by-products by headspace solid phase micro-extraction (SPME) - GC/MS.
2.3.2 Lactic acid fermentation of cheese whey using TC/\textit{L. bulgaricus} biocatalysts

The same process as above was followed for lactic acid fermentation of cheese whey (lactose $\sim$50 g/L) using the three immobilized TC biocatalysts.

2.3.3 Lactic acid fermentation of lactose and cheese whey using composite TC/alginate/\textit{L. bulgaricus} biocatalysts

The same process as above was followed for lactic acid fermentation of lactose synthetic media or cheese whey using the three composite TC/alginate/\textit{L. bulgaricus} biocatalysts.

2.3.4 Lactic acid fermentation of cheese whey using free \textit{L. bulgaricus} cells

Fermentations were also carried out as above using 1 g of harvested \textit{L. bulgaricus} culture (free cells) for comparison.

2.3.5 Alcoholic fermentation of glucose using TC/\textit{S. cerevisiae} biocatalysts and free cells

The three TC biocatalysts (mango, sal, and rice husk TCs with immobilized \textit{S. cerevisiae} cells) were used for repeated alcoholic fermentation batches of glucose media (composition as in §2.1.4.2 with $\sim$160 g/L initial glucose concentration). The fermentation was carried out initially at 30\degree C and then the temperature was decreased to 15 \degree C. Amounts of 1 g of harvested \textit{S. cerevisiae AXAZ-1} culture were added in flasks containing 400 mL of glucose medium and 5 g of the TC materials. All fermentations were carried out without agitation. After the completion of each fermentation (until a final density of 0.0-0.1\textdegree Be), the supernatant liquids were decanted and samples were collected for analysis after removal of suspended yeast cells by centrifugation at 5000 rmp for 10 min. The TC biocatalysts were used for a next batch fermentation after addition of fresh glucose medium. The samples were frozen until further analysis.

The fermented samples were analyzed for ethanol productivity by HPLC, and volatile by-products by GC/MS. For comparison, fermentations using free cells were also carried out.
2.3.6 Alcoholic fermentation of grape must using TC/S. cerevisiae biocatalysts and free cells

The three TC biocatalysts (mango, sal, and rice husk TCs with immobilized S. cerevisiae cells) were also used for repeated grape must fermentation batches (9.45 °Be or ~ 160.1 g/L initial sugar concentration) to evaluate the suitability of the biocatalysts for wine fermentation. The fermentations of grape must and fermented sample analyses were carried out using the same processes as described above.

Chapter 4 - Cold pasteurization of liquid foods

2.4.1 Cold pasteurization of water, skim and semi-skimmed milk

For the development of cold pasteurization processes for liquid foods, deliberately contaminated water and skim/semi-skimmed milk were used as model substrates. Water or milk, were pumped through the different TC filters in order to evaluate their efficiency for microbial load removal (yeast and bacteria). For this reason, water was inoculated with S. cerevisiae AXAZ-1 yeast cells (0.5 g/L; 70×10³ CFU/mL) and skim/semi-skimmed milk with L. bulgaricus (0.2 g/L; 28×10³ CFU/mL).

The system consisted of a cylindrical glass bioreactor (60 cm height; 7 cm internal diameter) packed with the different TC filters having various lengths (35 cm/1348 cm³ and 45 cm/1733 cm³) (Figure 23). A high accuracy peristaltic pump (Masterflex, Cole-Parmer, USA) was used at a flow rate of 2 L/day. The system was operated at 4°C in a domestic refrigerator. The effectiveness of the pasteurization process was evaluated by standard plate counting of the inlet and outlet liquid streams. Additionally, the optical density at 600 and 700 nm for L. bulgaricus and S. cerevisiae, respectively, was measured using a UV-VIS spectrophotometer (Jenway, UK). When the microbial load removal was reduced to 80-90%, regeneration of the filter was carried out by washing the filter with hot water (70-80°C). The operational stability of the system was monitored for 30-60 days.

2.4.2 Experimental set-up for continuous cold pasteurization

The dry weight of the each TC material used to prepare the filter for cold pasteurization was 400 - 450 g, depending on the concerned parameters, i.e. the filter length, therefore the liquid operating volume of the bioreactor was not stable (ranged between 1950 and 2050 mL). A graphic design of the continuous cold pasteurization system is provided below (Figure 23):
Figure 23. Continuous cold pasteurization system.

The continuous system consisted of a conical flask containing the contaminated liquid (water/ *S. cerevisiae* or milk/*L. bulgaricus*) placed on an agitation devise to maintain the cell suspension homogenous. The liquid was pumped in the inlet at the bottom of the bioreactor at a speed of 20 rpm/min to provide a flow rate of ~2 L/day. The liquid was therefore forced to pass upwards through the different TC filters (rice, mango and sal) which were fit in the bioreactor. The effluents were collected in a 3L-conical flask, which was connected with the outlet at the top of the bioreactor.

The inflowing liquid was prepared daily by suspending *S. cerevisiae* or *L. bulgaricus* cells in water or milk, respectively, as described below. The microbial load removal was evaluated by viable cell counts as well as optical density measurements of the influents and effluents every alternate day.

### 2.4.3 Viable cell counts

Ringer’s solution was prepared by dissolving 1 tablet of 1/4 strength in 500 mL of deionized water. Amounts of 9 mL of this solution were added in 12 test tubes, which were then autoclaved at 120°C for 15 minutes. Amounts of 1 mL of samples of water or milk obtained after the cold pasteurization process, were transferred to the 1st test
tube containing 9 mL of Ringer’s solution and the contents were mixed well. After that 1 mL of solution from the 1st test tube was transferred to the 2nd test tube containing 9 mL of the Ringer’s solution, and so on, until 12 decimal dilutions of the effluents were prepared. Amounts of 100 μL of these dilutions were spread plated on petri dishes containing potato dextrose agar and the dishes were then incubated at 30 or 37°C for yeast or bacteria counts, respectively. The colony forming units (cfu) on the petri dishes were counted to analyze the residual viable microbial load of the cold pasteurization effluents. The above same process was followed to calculate the microbial load in the influents.

2.4.4 Evaluation of filter efficiency, stability, and regeneration

Efficiency, stability, and reusability of the proposed cold pasteurization system are of great importance regarding its possible industrial application. Thus the bioreactor was fed for at least a period of 2-3 weeks, in order to examine its efficiency, operational stability and ease of regeneration. Prolonged operation of the system resulted in filter clogging and reduction of its microbial load removal efficiency. To regenerate the filter without constructing a new one, 5 liters of boiling water were passed through the bioreactor at the maximum pump upstream flow rate. This process was carried out at regular time intervals depending on the efficiency levels of the system.

**Chapter 5 - Analytical Methods**

**2.5.1 Proximate analysis of the Indian cellulosic materials**

Proximate analyses of the different plant species of Indian origin, i.e. mango (*M. indica* L.) and sal (*S. robusta* G.) sawdusts, and rice husk (*Oryza sativa* L.) were performed. All plant species were collected from Indian Jharkhand Forest in Ranchi area. The leaf, bark and husk of the plant species were oven dried and analyzed for moisture, nitrogen, crude protein, ash, relative water content (RWC) and lignin contents. The soil pH was determined. Graphical interpretation of the data was done using window stat software developed by Indostat, Hyderabad, India.

**2.5.1.1 Total nitrogen**

Total nitrogen and crude protein determination was carried out using the micro Kjeldahl distillation method. Amounts of 0.2 g of powered material from the three plant species were mixed with 3 g of catalytic mixture (copper sulfate + potassium sulfate in the ratio of 1:5) and 5 mL of conc. H₂SO₄. The mixture was digested at 420-
430°C until the color of the mixture become transparent. The mixture was cooled and the volume was made to 100 mL with distilled water. 5 mL alone were taken in micro Kjeldahl distillation apparatus and 5 mL of 40% NaOH solution was added. The ammonia which evolved was absorbed in 10 mL of boric acid solution (2.5 % w/v) with methyl red and bromocresol green as indicators. This was titrated against (N/70) H₂SO₄ solution.

\[
\text{Total nitrogen \%} = \frac{1.4 \times \text{volume of standard H}_2\text{SO}_4 \text{ solution (mL)}}{\text{Weight of sample (g)}}
\]

Crude protein was calculated by multiplication of total nitrogen by 6.25.

2.5.1.2 Ash

Amounts of 1 g of each powdered plant material were strongly heated for 5 h in a nickel crucible at 100°C over a Bunsen burner. After cooling the weight of the ash was measured and the total mineral content was calculated as:

\[
\text{Total Mineral content \%} = \frac{\text{Weight of ash}}{\text{Weight of sample (g)}} \times 100
\]

2.5.1.3 Soil pH

Amount of 10 g of soil samples were finely sieved through 2 mm sieves in a 50 mL beaker. 25 mL of distilled water was added and the mixture was stirred uniformly. The pH of the soil suspension was measured after 30 min using a pH-meter after standardizing with required buffer solution of known pH.

2.5.1.4 Lignin

Samples of 100 mg of the plant materials were dried at 60°C overnight and then were transferred in small Eppendorf tubes. Acetone was diluted in about 10:1 ratio with sterilized Milli Q water (SMQ) making a stock solution of about 20 mL. Then 2 mL of the acetone solution were added in each Eppendorf tube containing the sample. The tubes were covered with silver foils making small holes at the openings and were kept at 55°C in a thermomixer for about 24 h. The samples were then hydrolyzed with 1 mL of 72% sulfuric acid for 3 h at 20°C with continuous stirring. The content was diluted to 3% sulfuric acid with SMQ and autoclaved for 1 h. The residue was filtered and washed thrice with hot water, and then the residue was dried at 80°C in a glass crucible until the weight of the residue appeared constant. The dried residue was considered as the acid insoluble lignin. For estimation of the acid soluble lignin,
the filtrate was diluted 100 times with SMQ and OD was taken at 205 nm. Total percentage of lignin was calculated as the sum of the acid soluble and insoluble lignin. The following formula was used for the determination of acid soluble lignin:

\[
\text{Percentage acid soluble lignin} = \frac{OD_{205} \times \text{volume of filtrate (mL)}}{110 \times \text{weight of sample (mg)}}
\]

2.5.2 Thermogravimetric analysis/differential thermal analysis (TGA/DTA)

Thermo gravimetric analysis of the mango and sal sawdusts and rice husk samples was carried out under nitrogen atmosphere (flow rate: 200 mL/min) in a Perkin Elmer Diamond Thermogravimetric/Differential Thermal Analysis (TG/DTA) system. For the moisture removal, 10 mg of sawdust sample were placed in an alumina crucible and heated at 100°C until weight stabilization. Afterwards, the temperature was increased from 100 to 1000°C at heating rate of 10°C/min. The same analysis was conducted for the carbonized materials under air flow.

2.5.3 Scanning electron microscopy (SEM)

Samples of the TC materials before and after cell immobilization (with \textit{S. cerevisiae} or \textit{L. bulgaricus}) were examined by SEM to reveal information on their different porous microstructures. The samples were washed gently with deionized water under aseptic conditions and were freeze-dried and thermally dried overnight at 30°C. Afterwards all samples were coated with gold in a Blazers SCD 004 Sputter Coater for 3 min to increase their electron conductivity and were examined on a JEOL model JSM-6300 (Japan) SEM microscope.

2.5.4 Analysis of BET surface/porosity characteristics

The surface area and porosity characteristics of the TCs were evaluated by measuring the amount of N\textsubscript{2} adsorbed and desorbed onto the materials over a wide range of relative pressures. The N\textsubscript{2} measurements were carried out at 77.35 K using the Micromeritics Tristar Surface Area and Porosity Analyzer. Before the measurements the samples were dried overnight at 40°C and degassed at 100°C for 2h in special glass cuvettes under vacuum (10 μm Hg) in order to remove any adsorbed species. Then, the cuvettes were submerged into a liquid nitrogen bath (77.35 Kelvin). The analyzing gas (N\textsubscript{2}) diffused within the interior of the chambers containing the samples and was absorbed uniformly by them. The extent of adsorption is proportional to the porosity of the analyzed sample. The Brauner-Emmet-Teller (BET) specific surface area was calculated by the BET equation (BRUNAUER, EMMETT & TELLER, 1938). Pore size distribution (pore volume and average pore diameter) of the samples was estimated by the Barrett-Joyner-Halenda
2. Experimental Part

2.5.5 SPME GC/MS analysis of volatile compounds

The volatile compounds of the alcoholic and lactic acid fermentation products were determined by means of gas chromatography-mass spectroscopy. More specifically, the volatiles were isolated by headspace solid phase micro-extraction (SPME) method. The fiber used for the absorption of volatiles was a 2-cm fiber coated with 50/30 mm divinylbenzene/carboxen on poly-dimethyl-siloxane bonded to a flexible fused silica core, (Supelco, Bellefonte, PA, USA). The conditions of headspace-SPEM sampling were as follows: 10 mL liquid sample, 3 g NaCl and internal standard (methyl octanoate) were transferred into a 20-mL head space vial fitted with a teflon-lined septum sealed with an aluminum crimp seal. The contents were magnetically stirred for 5 min at 60°C, and then the fiber was exposed to the headspace for 45 min. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the gas chromatograph in the splitless mode, at 240°C for 3 min. Before each analysis, the fiber was exposed to the injection port for 5 min to remove any volatile contaminants.

GC/MS analysis was performed on a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer. Helium was used as carrier gas (1.8 mL/min). Separation of compounds was performed on a capillary column (Supelco CO Wax-10 60m, 0.32 mm i.d., 0.25 µm film thickness). Oven temperature was programmed at 35°C for 6 min and then it was raised to 60°C with a rate of 2°C/min, held constant for 5 min, raised to 200 and 250°C with a rate of 5 and 25°C/min respectively. Finally, it was held at 250°C for 6 min. The injector and interface temperatures were set at 240°C. The mass spectrometer was operated in the scan range 45-400 m/z. Identification of the compounds was effected by comparing the Kovats’ retention indices based on the even n-alkanes (C10-C24) with those of standard compounds and by the literature. Kovats’ retention indices, and MS data with those of standard compounds and by MS data obtained from NIST107, NIST21 and SZTERP libraries. Semi-quantitative analysis was performed by dividing the peak area of a compound with the peak area of the internal standard and multiplying the result with the concentration of the internal standard (10 µg/L).

2.5.6 HPLC analysis of organic acids

Organic acids were analyzed on a HPLC system (Jasco Inc., Japan) LC-2000 Series equipped with a size-exclusion organic acid analysis column (Aminex HPX-87H, 300x 7.8 mm i.d., 9 µm particle size, Bio-rad, France) fitted in a CO-2060 PLUS column oven. A PU-2089 pump, an AS 2050 PLUS autosampler and a MD-2018 Photodiode array detector (set at 210 nm) were employed. Isocratic separation at 50°C, with a flow rate of 0.6 mL/min and 0.008 N H2SO4 as mobile phase, was performed. The detector signals were recorded and analyzed by ChromNav software. Amounts of 20 µL of the samples were filtered through a 0.2 µm nylon filter. Standard solutions of acids (Sigma-Aldrich Ltd) in 3D water were prepared at various concentrations for
quantification.

2.5.7 HPLC analysis of sugars and ethanol

Sugars (glucose, fructose, sucrose, lactose and galactose) and ethanol in the fermented products were determined by HPLC on a Shimadzu LC-9A HPLC system consisting of a Shim-pack SCR-101N column, an LC-9A pump, an RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit. Three times distilled water was used as the mobile phase (0.8 ml/min), and 1-butanol (0.1% v/v) was used as an internal standard. The column temperature was 60°C. The sample dilution was 1% v/v, and the injection volume was 40 μL. Determinations were done based on standard curves.

2.5.8 FT-IR analysis

Samples of the TC filters were collected after the end of the cold pasteurization processes. Pellets of 2 mg of the TCs were prepared by mixing with 200 mg KBr (spectroscopic grade). Infrared spectra (4000-400 cm\(^{-1}\)) were recorded on a Perkin-Elmer (Massachusetts, USA) spectrometer with a resolution of 4 cm\(^{-1}\) and 10 scans per sample. Background spectrum was obtained using a TC/KBr pellet.

2.5.9 X-Ray Diffraction

In order to determine the crystalline nature of the TC samples, the XRD patterns were obtained on an Enraf Nonius FR590 diffractometer with CuKα radiation generation. The intensity was measured between 2θ of 5-60°. The crystallinity index (CI) was calculated from the heights of the 200 peak (I\(_{002}\), 2θ=22.6°) and the intensity minimum between the 200 and 110 peaks (I\(_{am}\), 2θ=18°) using the Segal method (GUMUSKAYA, USTA & KIRCI, 2003). I\(_{002}\) represents both crystalline and amorphous material, whereas I\(_{am}\) represents the amorphous material.

\[
C_I(\%) = \left(\frac{I_{002} - I_{am}}{I_{002}}\right) \times 100
\]  

(1)

The crystallite size was estimated from the full width at half-maximum intensity (FWHM) of the reflection (200) using the Scherrer equation (FRENCH & CINTRON, 2013):

\[
\beta_{hkl} = \frac{K\lambda}{L_{hkl}\cos\theta_{hkl}}
\]  

(2)
where $\beta$ is the breadth of the peak of a specific phase, $K$ is a constant that varies with the method of taking the breadth ($K=0.94$), $\lambda$ is the wavelength of incident X-rays ($\lambda=0.15418$ nm), $\theta$ is the centre angle of the peak, and $L$ is the crystallite length (size).

2.5.10 Statistics

All analyses for fermentations were carried out in triplicate and the results are presented as mean values $\pm$ standard deviations. The significance of differences in the means of various data groups was checked by One-way Analysis of Variance (ANOVA) at the 5% level of significance. Graphical interpretations for fermentations and cold pasteurization of water and milk were done by Microcal Origin software.
3. Results & Discussion

Chapter 1 - Rationale of the thesis

Delignified cellulose (TC) from wood sawdust is a porous material that can be produced by heat treatment with aqueous NaOH solution. The TC material has earlier been proved to be an excellent support for cell immobilization and promoter of fermentation processes, like wine making, brewing, lactic acid production, etc., leading to improvement of productivity and final product quality (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014; KOUTINAS et al., 2012). Hence, the present investigation involves (i) the use of nano/micro porous TC materials of Indian origin (ii) as promoters of lactic acid and alcoholic fermentation processes and (iii) as natural biofilters (traps for microflora) for liquid food cold pasteurization as alternatives to conventional thermal-involving pasteurization processes.

3.1.1 Cold pasteurization with nano/micro porous TC filters

Pasteurization is a technique that is widely used in industrial food production, which involves thermal treatment of the food to remove all vegetative forms of microorganisms. Nutritional value and quality standards for many industrially produced food depends on the temperature and duration of pasteurization. However, such treatments usually degrade the sensory and nutritional properties of the food, causing undesirable effects such as lipid oxidation, destruction of ascorbic acid and other vitamins, loss of volatile compounds, development of off-flavors and formation of melanoidines (ZULUETA et al., 2013; QIN et al., 1995; VEGARA et al., 2013; SKREDE & DURST, 2000).

To overcome the drawbacks of thermal treatment without decreasing its effectiveness in removing spoilage and pathogen microorganisms, several other techniques have been proposed, as described in the Chapter 8 of the Introduction section, but with no significant effects on food quality and cost effectiveness. The present investigation targets in preventing the undesirable effects of high temperature processing, by proposing a simple continuous filtration technique at refrigeration temperature.

Drinking water available in open environment and milk before pasteurization, contain many undesirable and naturally occurring microbial floras able that can cause adverse effects on human health. A common method employed nowadays for
that purpose, is the filtration through various membranes. However, membranes have to be replaced often due to fouling and this renders the process inapplicable for small scale industries, for example, low budget wineries. *Acetobacter* species, depending on the strain, and yeasts are quite resistant to relatively high levels of SO$_2$ and can be active at levels below 200 mg/L (SCHIMZ, 1980). This investigation aims to develop a novel method of cold pasteurization based on porous TC materials (as shown in Figure 23) using water and milk as model substrates, for creating the basis for application to other types of liquid foods such as juices, wines, etc.

TC is a natural inert product, non-toxic, easily available, and of low cost. During the delignification of cellulose, lignin, which is a phenolic polymer (Figure 12), is removed creating pores and tubular structures. Microbial cells were found to be entrapped into this porous structure and thus be removed from a liquid (i.e. water and milk). The extracted lignin polymer can be utilized further to produce other food grade natural microbial filters, by agglomeration with other polymeric materials, and then be removed by alkaline treatment thus leaving behind new pores and tubes in their matrices. The nano/micro porous TC produced by the delignification of lignocellulosic materials is proposed as filter for cold pasteurization to remove the microbiological load of liquids of pharmaceutical industries and liquid foods like drinking water, and milk. The cold pasteurization process for drinking water and milk was carried out in a continuously operating glass bioreactor packed with the nano/micro TC filter, which was packed in a thin perforated nylon fabric covered with stainless steel covers like in the compression moldings (Figure 23).

Furthermore, cellulose is a cheap and abundant material available worldwide and especially in the developing countries with less access to clean drinking water, poverty, pollution and lack of organized infrastructures. The application of this ubiquitous biomass that is naturally found in the environment can solve the major problems related to clean and drinking water availability. From conclusions of post-investigation analyses, Indian cellulose contains pores and tubes of very broad range sizes, from nano to microscales that could be exploited for cold pasteurization of contaminated Indian water.

### 3.1.2 Promotion of biocatalysis by nano/micro porous TC

The use of TC of Indian origin to advantage bioprocessing is similarly an area of increased scientific interest. The development of bioprocesses for synthesis of eco-friendly materials is considered important in the present investigation in order to further expand their bio-chemical applications. Nowadays, a variety of green materials, including nanoparticles, with well-defined chemical composition, size, and morphology, have been characterized and synthesized by different methods and their applications in many cutting-edge technological areas have been explored. Such an approach is the promotion of biocatalysis (fermentation technologies) by the Indian origin nano/micro TCs. The proposed biocatalysts are of food grade purity and their good mechanical strength, as plain materials or composites, during the bioprocess application indicated increased operational stability and reusability and, thus, cost efficiency.
Figure 24. Experimental methodology
3. Results & Discussion

3.1.3 Experimental methodology

The morphological structure of the nano/micro porous TC materials was performed by SEM and by porosimetry analysis to study their effective surface area and porous structure. Their efficiency for liquid food cold pasteurization and on promotion of lactic acid and alcoholic fermentations was examined by evaluation of microbial load removal and fermentation rate/product quality, respectively. The fermented products were examined by HPLC, GC and GC-MS analysis. The microbiological analysis was done by suitable plate counts for yeasts, and bacteria and OD measurements. Comparison of microbial load removal efficiency was done for both the proposed methods and conventional pasteurization methods. The results were statistically treated, and sensory evaluations were also performed. A graphic description of the experimental methodology followed in this thesis is presented in Figure 24 (and Table 9). The impact on food quality and on process cost, which define the feasibility for commercial applications of the proposed biocatalysts and processes were also evaluated and discussed.

3.1.4 Why Indian origin cellulosic materials

The need for innovative and sustainable technologies in the area of food bioprocessing and fermentation technology has brought upon a great interest on the use of cheap renewable resources. Rice husk is one of the available biomass that occupies a strong position among a variety of agricultural wastes not only in terms of amounts produced worldwide, but also because of its unique chemistry-related features. The production of silicon-based materials and active carbon are some of the known wide applications of rice husk. The husk ash contains potassium, sodium, magnesium, calcium, iron, and phosphorus, as well as considerably smaller quantities of copper, iron, manganese, etc. A large quantity of the world production of rice husk and rice husk ash, remain as unused waste materials. As a result, a large number of possible industrial applications of rice husk ash has been investigated. Extensive research has been carried out on the preparation, properties and applications of rice husk ash during the last three decades and many scientific investigations and patents have been published on this subject (SOLTANI et al., 2014). However, its utilization as porous filters for cold pasteurization is novel.

Forestry lignocellulosic wastes have long been used as good raw materials for the preparation of activated carbon. Indian origin wood sawdust and rice husk are such materials that can be used as novel biodegradable micro/nano porous cellulosics after delignification. The northern parts of India are mostly covered with dense forests and the timber industries in these areas are one of the major businesses employing over 30,000 people. There have been several reports of lung damage attributed to inhaled wood dust in timber workers but no clinical reports of their harmful effects are available. Sheesham (Dalbergia sissoo), mango (Mangifera indica) and sal (Shorea robusta) are the commonest woods used as furniture and building materials, whereas rice husk is more commonly used by farmers for various domestic purposes including fire for cooking and room heaters. The bark and other tannin-rich materials are also good sources for organic adsorbents. Bark from where
the wood sawdust is extracted is a solid waste of timber industry and is used as possible adsorbent; however the main problem associated with tannin-containing materials is discoloration of the water due to soluble phenols (EDGEHILL & LU, 1998). In this thesis chemical pretreatment (delignification) was carried out before use of these materials (i.e. wood sawdust and rice husk) for further bioprocessing. The scientific exploitation of these naturally available lignocellulosic biomasses of Indian origin has not yet received much attention. Thus, a bulk utilization of Indian nano/micro porous TC for food bioprocessing applications can also solve the problem of its disposal in the timber industry and create added value.

Chapter 2 – Characterization of Indian TCs

3.2.1 Proximate analysis

In order to use various origin Indian TCs for bioprocess development, it was though appropriate to analyze the proximate chemical composition of the raw materials. Therefore, chemical characteristics of the three plant species (\textit{S. robusta}, \textit{M. indica} & \textit{O. sativa}) were analyzed and the results are summarized in Tables 10-12 and Figure 25-Figure 29 below.

Specifically, the percentages of ash, moisture, relative water content, nitrogen and crude protein present in samples of leaf, bark & husk of the different plant species are illustrated in Figure 25-Figure 29 below.

The \textit{O. sativa} leaves (Figure 25) and husk (Figure 26) contained higher amounts of moisture, ash and crude protein compared to the leaves and bark of \textit{S. robusta} and \textit{M. indica}.

The \textit{S. robusta} leaves (Figure 27) contained higher amounts of moisture, ash and crude protein compared to its bark.

Regarding \textit{M. indica} (Figure 28), higher amounts of ash and crude protein, but lower moisture, were found in leaf compared to its bark.

Finally, the \textit{O. sativa} leaves (Figure 29) contained lower amounts of moisture, but higher amounts of ash and crude protein compared to its husk.
Figure 25. Proximate analysis of dry leaf extracts.

Figure 26. Proximate analysis of bark/husk samples.
3. Results & Discussion

**Figure 27.** Comparative analysis of dry leaf (L) & bark (B) extracts of *S. robusta*.

**Figure 28.** Comparative analysis of dry leaf (L) & bark (B) extracts of *M. indica*. 
In Table 10 & Table 11 the pH, RWC, ash, moisture, total nitrogen and crude protein contents in leaf and bark/husk samples, respectively, of *O. sativa*, *S. robusta* and *M. indica* are presented. Table 10 indicates the percentage of moisture in leaf sample to be about equal in *O. sativa* as compared to *M. indica* and *S. robusta*, whereas RWC to be higher only in *S. robusta* in comparison to *O. sativa* and *M. indica*. The relative water content is associated with protoplasmic permeability in cells which regulates loss of water and dissolved nutrients that result in early senescence of leaves. It plays an important role in photosynthetic carbon fixation with the reducing power directly proportional to its concentration (WAHID & BABU, 2005).

From Table 11 the presence of RWC is recorded in higher values in *O. sativa* husk and *M. indica* and *S. robusta* bark. The higher concentration of protein content in *O. sativa* husk reflects the higher content of protein present in the TC that comes from rice husk. The presence of high protein content may also contribute to more fermentative biocatalysts that are produced by encapsulation of cells in TC, due to higher availability of amino acids and assimilable nitrogen for the yeast or bacteria species. Likewise, *O. sativa* contains more minerals (ash) that could play an inhibiting or promotional role on cell activity.
### Table 10. Mean values of proximate analysis parameters of leaf samples.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>pH</th>
<th>RWC (%)</th>
<th>Ash (%)</th>
<th>Moisture (%)</th>
<th>Nitrogen (%)</th>
<th>Crude protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. robusta</em></td>
<td>5.6 ±0.1</td>
<td>4.80±0.26</td>
<td>4.0±0.11</td>
<td>54.4±2.9</td>
<td>1.90±0.22</td>
<td>11.87±0.84</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>6.1±0.6</td>
<td>0.75±0.13</td>
<td>12.2±0.17</td>
<td>47.0±2.3</td>
<td>1.35±0.17</td>
<td>8.42±0.48</td>
</tr>
<tr>
<td><em>O. sativa</em></td>
<td>5.5±0.1</td>
<td>0.40±0.20</td>
<td>23.8±0.35</td>
<td>59.7±1.7</td>
<td>2.91±0.19</td>
<td>18.18±1.01</td>
</tr>
</tbody>
</table>

**CD at 5 %** 0.33610 0.70271 0.81351 8.12123 0.67091 2.79450

### Table 11. Mean values of proximate analysis parameters of bark or husk samples.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>pH</th>
<th>RWC (%)</th>
<th>Mineral (%)</th>
<th>Moisture (%)</th>
<th>Nitrogen (%)</th>
<th>Crude protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. robusta</em></td>
<td>5.6±0.1</td>
<td>86.8±0.9</td>
<td>0.04±0.00</td>
<td>51.5±0.8</td>
<td>0.39±0.06</td>
<td>2.43±0.15</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>6.1±0.6</td>
<td>90.0±0.9</td>
<td>0.028±0.00</td>
<td>59.0±1.5</td>
<td>0.02±0.02</td>
<td>1.62±0.28</td>
</tr>
<tr>
<td><em>O. sativa</em></td>
<td>5.5±0.1</td>
<td>90.4±0.4</td>
<td>0.13±0.00</td>
<td>59.7±1.7</td>
<td>1.46±0.19</td>
<td>9.12±0.48</td>
</tr>
</tbody>
</table>

**CD at 5 %** 0.33610 0.33610 2.70873 0.00312 9.01978 0.39858

*Tables 11 & 12: Statistical analysis was carried out using window stat software developed by Indostat, Hyderabad, India.*
3.2.2 Micro/nano structure of Indian TCs

The different cellulosic materials of Indian origin (mango and sal sawdust and rice husk), were sieved for uniformity and then they were delignified by alkaline treatment. The occurring materials were drained and dried or stored in wet form depending on the subsequent use. A photo of dried wood TC is shown in Figure 30. The concentration of alkaline solution (i.e. 1 % NaOH) used for delignification was adequate for efficient removal of lignin from the cellulosic matrix. Alkaline treatment of lignocellulosic biomass is also commonly used to improve subsequent saccharification by enzymes due to removal of lignin and uronic constituents that are responsible for inhibition of cellulase binding and by cleaving the lignin bonds with hemicelluloses and cellulose (CHO et al., 2013).

![Figure 30. Dried delignified cellulosic material (TC).](image)

The solubilization and removal of lignin during the alkaline treatment is obvious by the dark color of the delignification mash (Figure 31). Figure 32 shows the wet delignified TC material before (dark brown) and after (yellowish-brown) washing with hot water.

![Figure 31. Wood sawdust delignification mash.](image)
3. Results & Discussion

3.2.3 SEM study of the TCs

The effects of alkaline treatment on the structure of the prepared TCs were examined by SEM, showing extensive microporous, mainly tubular, structures (Figure 33), similar with those of Greek origin wood sawdust TCs used in previous studies (KOUTINAS et al., 2012). The wood-derived tubes are horizontal and parallel, and are attached to each other forming bundles of tubes. Increased porosity can be observed by smaller random pores formed vertically on the internal surface of the wood tubes and other surfaces, as shown in Figure 33. This tubular and porous structure facilitates microbial cell entrapment and may also facilitate entrapment of enzymes creating novel biocatalysts suitable for use in fermentation processes, as well as filters for cold pasteurization and as “storehouses” for enzymes.

3.2.4 Lignin estimation

After the delignification of Indian cellulosic materials, the TCs were analyzed for lignin by the Klason method and the results are presented in Table 12. This was done in order to estimate the amount of lignin that was removed by the applied alkaline delignification treatment. The total lignin content usually present in rice husk depending upon the species is 11-23 %, whereas the total lignin content in mango wood is 27-45.8 %, and in sal wood 22-39.7 % (Forest research institute, Ranchi INDIA; ONYANGO et al., 2011; REDDY et al., 1997).

The total percentage of lignin was calculated as the sum of the acid soluble and insoluble lignin.
Figure 33. SEM pictures of the delignified (a) mango, (b) rice and (c) sal TCs.
### Table 12. Lignin content in the Indian cellulosic materials before and after delignification.

<table>
<thead>
<tr>
<th>Plant Sample</th>
<th>1st OD</th>
<th>2nd OD</th>
<th>OD Mean Difference</th>
<th>Starting sample (mg)</th>
<th>Acid soluble lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before Delignification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. sativa</td>
<td>0.751</td>
<td>0.694</td>
<td>0.722</td>
<td>30</td>
<td>19.6 %</td>
</tr>
<tr>
<td>S. robusta</td>
<td>1.762</td>
<td>1.711</td>
<td>1.733</td>
<td>20</td>
<td>31.4 %</td>
</tr>
<tr>
<td>M. indica</td>
<td>2.305</td>
<td>2.261</td>
<td>2.283</td>
<td>20</td>
<td>41.4 %</td>
</tr>
<tr>
<td><strong>After Delignification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. sativa</td>
<td>0.110</td>
<td>0.108</td>
<td>0.100</td>
<td>30</td>
<td>2.7 %</td>
</tr>
<tr>
<td>S. robusta</td>
<td>0.675</td>
<td>0.670</td>
<td>0.672</td>
<td>20</td>
<td>12.2 %</td>
</tr>
<tr>
<td>M. indica</td>
<td>0.784</td>
<td>0.780</td>
<td>0.782</td>
<td>20</td>
<td>14.2 %</td>
</tr>
</tbody>
</table>

The Table above shows that the amount of acid soluble lignin before the delignification of rice husk was 19.6%, which concludes that 3.4% were the acid insoluble lignin. Comparatively, in sal sawdust 31.4 % was the acid soluble lignin and 8.3% was the acid insoluble lignin, whereas for the mango sawdust the acid soluble lignin was around 41.4 % concluding that 4.4 % was the acid insoluble lignin.

Similarly the amount of acid soluble lignin present in the lignocellulosic samples after the delignification process was reduced to 2.7% for rice husk, 12.2 % for sal sawdust and 14.2% for mango sawdust. This concludes a fair amount of lignin being removed during the delignification process leaving behind a porous structure of cellulose and hemicelluloses.

The delignification is therefore necessary to remove lignin, which may be toxic to microorganisms, leave undesirable residues and cause discoloration in the target food products. Also it is necessary to increase the materials porosity and facilitate entrapment of microbial cells and enzymes, as discussed above, or promote the rate of fermentation processes as discussed in previous studies (sugar pump theories) (KOUTINAS et al., 2012; GANATSIOS et al., 2014).

Taking into account that the Greek wheat straw, used in a previous similar study (KOUTINAS et al., 1981), contains 16% total lignin instead of 41.4 % it is obvious that delignification of mango sawdust will lead to a material with increased porosity and therefore higher specific surface area as compared with Greek straw. The higher lignin content means increased lignin as useful by-product and therefore reduction of TC production cost.
3.2.5 Porosimetry analysis (BET surface)

The extent of N\(_2\) adsorption is proportional to the porosity of the analyzed sample. To increase the porosity of the delignified rice, mango and sal TCs, they were also carbonized as described below. All materials were analyzed for BET surface area (m\(^2\)/g), cumulative pore volume (cm\(^3\)/g) and average pore diameter (Å), before and after delignification and after carbonization (Table 13, Table 14 & Table 15, respectively).

After the delignification process it is shown that the rice husk had a five-fold increase in surface area (6.6 m\(^2\)/g) and at least three-fold higher pore volume compared to mango sawdust (1.05 m\(^2\)/g) and sal sawdust (0.6 m\(^2\)/g). Therefore, the delignification caused an increase of 50-110\% of BET surface. The average pore diameter for rice husk was found in the range of 100 Å indicating in relation to pore volume, that nanopores posse more than 2\% of the volume of TC as compared to the non-delignified cellulosics. Mango TC exhibited similar surface characteristics to other porous materials that have been previously used for bioprocess applications, such as γ-alumina (1.40 m\(^2\)/g) (KOUTINAS et al., 1991; KOURKOUTAS et al., 2004; SYNGIRIDIS et al., 2013). However, the average cumulative pore volume of all TCs was in the range 0.010-0.002 cm\(^3\)/g being smaller compared to γ-alumina (0.45 cm\(^3\)/g). The results show that the TCs have increased porosity characteristics after the delignification process, depending on their plant origin, and may be more suitable for bioprocessing such as cold pasteurization, enzyme storage carriers and immobilized cell biocatalysts.

### Table 13. Porosimetry parameters of TC materials before delignification.

<table>
<thead>
<tr>
<th>Delignified cellulose</th>
<th>Rice husk</th>
<th>Mango</th>
<th>Sal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area (m(^2)/g)</td>
<td>4.03</td>
<td>1.05</td>
<td>0.69</td>
</tr>
<tr>
<td>Pore volume (cm(^3)/g)</td>
<td>0.020</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>Average pore diameter (Å)</td>
<td>190</td>
<td>146</td>
<td>132</td>
</tr>
</tbody>
</table>

### Table 14. Porosimetry parameters of TC materials after delignification.

<table>
<thead>
<tr>
<th>Delignified cellulose</th>
<th>Rice husk</th>
<th>Mango</th>
<th>Sal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Area (m(^2)/g)</td>
<td>6.6</td>
<td>2.19</td>
<td>1.04</td>
</tr>
<tr>
<td>Pore volume (cm(^3)/g)</td>
<td>0.018</td>
<td>0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>Average pore diameter (Å)</td>
<td>104</td>
<td>174</td>
<td>156</td>
</tr>
</tbody>
</table>
3. Results & Discussion

Chapter 3 - Carbonization of TCs

3.3.1 Effect of carbonization on TC porosity characteristics

In order to increase the porosity of the Indian TC materials after delignification, they were carbonized by high temperature treatment (250 and 350°C for 6 and 12 h, respectively, under controlled pressure and nitrogen flow). However, to select the suitable temperature for carbonization of the TCs, it was necessary to examine the wood sawdust and rice husk thermal behavior by Thermogravimetric Analysis (TGA). The results of the porosimetry analysis of the carbonized TCs are presented in Table 15 & Table 16.

Figure 34 (up) shows TGA data information on mass change behavior of all TCs, during increase of temperature up to 1000°C. It is indicated that near to 350°C the highest weight loss was observed for all types of TCs. These results were confirmed by Differential Thermal Analysis (DTA) as shown in Figure 34 (down). The first stage (temperature below 100°C) corresponds to the moisture evolution due to evaporation. The second stage (above 220°C to approximately 310°C) is mainly due to the thermal decomposition of hemicelluloses and some portion of lignin. The last stage in the high temperature range (310-400°C) is associated with the degradation of cellulose and lignin. In this temperature range, almost all cellulose components were decomposed. The residual lignin still present in the TCs after the chemical delignification pretreatment extended the decomposition and carbonization temperature range starting from 200°C up to 700°C.

Figure 35 (a & b) illustrates SEM images of carbonized mango and rice TCs, showing similar tubular structures after carbonization. However, it can be observed that the walls of the tubes appear foam like (spongy), which explains the substantial increase of BET surface.

The mango and sal sawdust TCs presented similar and higher thermal stability compared to rice husk TC, as the abrupt changes in weight loss were observed at the temperature of 350°C. Rice husk presented similar thermal stability behavior up to 300°C with the wood materials, but weight loss was more severe beyond that temperature.

Concluding, the carbonization temperature and duration of heating the TCs can substantially increase the porosity characteristics and mainly the surface area, depending on the origin of the TCs. Carbonization of TCs at 350°C for both 6 and 12 h showed higher increase in the BET surface in the range 10-16 m²/g; but with increase in BET surface there was a decrease in the mechanical strength of the TC material. The texture was similar to charcoal but more brittle in touch and more easily dissolved when in contact with liquid media.

This work comprises an approach for more effective utilization of the delignified TCs and carbonized TCs as nano/microporous adsorbent materials for water treatment, dyes, heavy metals and other toxic chemicals in industrial effluents.
Figure 34. TGA (up) and DTA (down) analysis of rice husk, mango and sal TCs for 12 hours.
3. Results & Discussion

Figure 35. SEM pictures of carbonized TCs at 350°C. (a) Mango and (b) Rice husk.

Table 15. Porosimetry parameters of carbonized TCs at 250 °C.

<table>
<thead>
<tr>
<th>Heating Time (h)</th>
<th>Delignified cellulose</th>
<th>Rice husk</th>
<th>Mango</th>
<th>Sal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BET surface (m²/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.4</td>
<td>4.0</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pore volume (cm³/g)</td>
<td>0.01</td>
<td>0.007</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Average pore diameter (Å)</td>
<td>158</td>
<td>156</td>
<td>175</td>
</tr>
<tr>
<td>12</td>
<td>BET surface (m²/g)</td>
<td>7.2</td>
<td>6.1</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>Pore volume (cm³/g)</td>
<td>0.3</td>
<td>0.15</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Average pore diameter (Å)</td>
<td>150</td>
<td>197</td>
<td>165</td>
</tr>
</tbody>
</table>
3. Results & Discussion

Table 16. Porosimetry parameters of carbonized TCs at 350°C.

<table>
<thead>
<tr>
<th>Heating Time (h)</th>
<th>Delignified cellulose</th>
<th>Rice husk</th>
<th>Mango</th>
<th>Sal</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>BET surface (m²/g)</td>
<td>10.0</td>
<td>8.4</td>
<td>8.7</td>
</tr>
<tr>
<td>6</td>
<td>Pore volume (cm³/g)</td>
<td>0.06</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>Average pore diameter (Å)</td>
<td>231</td>
<td>199</td>
<td>200</td>
</tr>
<tr>
<td>12</td>
<td>BET surface (m²/g)</td>
<td>12.2</td>
<td>11.3</td>
<td>16.2</td>
</tr>
<tr>
<td>12</td>
<td>Pore volume (cm³/g)</td>
<td>0.07</td>
<td>0.04</td>
<td>0.2</td>
</tr>
<tr>
<td>12</td>
<td>Average pore diameter (Å)</td>
<td>262</td>
<td>166</td>
<td>265</td>
</tr>
</tbody>
</table>

Table 17. Elemental analysis (SEM-EDS) of carbonized TCs at 350°C for 12 h.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Mango (%wt)</th>
<th>Sal (%wt)</th>
<th>Rice (%wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>37.68</td>
<td>22.73</td>
<td>9.30</td>
</tr>
<tr>
<td>O</td>
<td>39.79</td>
<td>57.82</td>
<td>58.18</td>
</tr>
<tr>
<td>Na</td>
<td>5.11</td>
<td>-</td>
<td>4.66</td>
</tr>
<tr>
<td>Mg</td>
<td>1.69</td>
<td>0.27</td>
<td>0.85</td>
</tr>
<tr>
<td>Ca</td>
<td>11.26</td>
<td>19.18</td>
<td>2.08</td>
</tr>
<tr>
<td>K</td>
<td>0.77</td>
<td>-</td>
<td>0.22</td>
</tr>
<tr>
<td>Si</td>
<td>1.84</td>
<td>-</td>
<td>24.60</td>
</tr>
<tr>
<td>Fe</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Al</td>
<td>0.92</td>
<td>-</td>
<td>0.14</td>
</tr>
<tr>
<td>Totals</td>
<td>100</td>
<td>100</td>
<td>100.00</td>
</tr>
</tbody>
</table>

The elemental analysis by SEM-EDS of carbonized TCs (Table 17) showed existence of calcium carbonate and hexagonal graphite in all TCs. Low concentration of Na₂O and MgO was found only in mango and rice TCs. The carbonized TCs were found to be consisting of mostly organic matter that was formed during pyrolysis under the absence of oxygen and presence of nitrogen. Table 18 shows the presence of nanotubes in all types of TCs that ranged between 27-51%. The pore size distributions in nanoscale are presented in Figure 36, illustrating that nanotubes of carbonized sal TC were much more as compared with pine TC, rice husk TC and mango TC.
Table 18. Nanotube pore volume percentage in carbonized TCs after carbonization at 350°C.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Time of treatment (h)</th>
<th>Total pore vol. (cm$^3$/g)</th>
<th>Nano pore vol. (cm$^3$/g)</th>
<th>% of nano pore vol. in total vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>6</td>
<td>1.4</td>
<td>0.43</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.5</td>
<td>0.44</td>
<td>28</td>
</tr>
<tr>
<td>Mango</td>
<td>6</td>
<td>1.2</td>
<td>0.33</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.94</td>
<td>0.25</td>
<td>27</td>
</tr>
<tr>
<td>Sal</td>
<td>6</td>
<td>0.90</td>
<td>0.27</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.30</td>
<td>0.66</td>
<td>51</td>
</tr>
</tbody>
</table>

Figure 36. Pore size distribution of carbonized materials at 350°C for 12 h.
Chapter 4 – Composites based on biopolymers and Indian TCs

3.4.1 Preparation of composites

Composite materials based on the Indian TCs blended with Ca-alginate and PLA were prepared. The resulting material is presented in Figure 37(a,b,c) showing TC/PLA/Alginate spherical beads of uniform size and the fibrous structure of the cross sections. The composite beads were highly porous and brittle when pressed.

Freshly prepared beads are generally spherical in shape, depending on the distance between the tip of the pipette or syringe, from where the dispersion takes place, and the solidifying solution, as well as on the stirring speed. The beads are flattened when they are dispersed from a height more than 6 cm. Drying of the beads may also affect their size and shape as during drying they usually shrink significantly (ASLANI & KENNEDY, 1996). It has also been reported that by increasing the solidifying solution concentration (e.g. calcium chloride) the obtained beads are smaller (SRIAMORNSAK et al, 1999). Figure 37(d,e) shows freeze-dried alginate beads and their cross section (SMRDEL et al., 2008).

Figure 37. SEM of mango TC/PLA/Alginate composite beads (a) and their cross sections (b & c); (d) freeze-dried alginate beads and (e) their cross section (SMRDEL et al., 2008).
Chapter 5 – Spectroscopic analysis of the TCs

FTIR spectra of the three different plant source TCs (mango, sal and rice) after delignification and after carbonization are shown in Figure 38-Figure 40. The data analysis presented in Table 19-Table 21 provides structural information on the molecular characteristics of the TCs after delignification and carbonization. Samples of TC were analyzed using a TC/KBr pellet as background in order to detect differences in wavelengths that could be used to identify the presence of compounds adsorbed during cold pasteurization of specific liquid foods like wines, beers, and fruit juices. Cold pasteurization of drinking water was easier to determine the presence of minerals and ions through elemental analyses.

3.5.1 FTIR analysis of rice husk TC

FTIR analysis of rice TCs after delignification and carbonization is presented in Table 19 showing variations in the presence of wave lengths and their effects on the band origin. Specifically, the FTIR spectra of rice TC show bands of OH, CH₂, C=O, C=C, C-O-C, C-H which are adapted mainly as per the molecular structure of carbohydrates. However, the result of carbonized TC shows the main bands of Si-OH silanol, ring structure of SiO₄ tetrahedra, and Si-O-Si. Likewise, C-O, C=O and bending of the O-H bond with small fraction of residual organic functionalities from CH₃CH₂ are also observed. Thus conclusion of this structure is that the carbonized rice TC contains mainly inorganic skeleton comprised of mainly silanol and SiO₄ tetrahedra and organic mass with functionalities of CH₃CH₂ containing OH and C=O groups. Therefore, carbonized TC retained their initial porous structure (as shown by SEM) which mostly consisted of siliceous, inorganic matter with a residual amount of organic matter.

More specifically and in details the FT-IR spectra of rice TC after delignification and after carbonization present considerable changes, especially in the region 1800-400 cm⁻¹ known as the fingerprint region. It is very clear that after carbonization the rice TC has extensively decayed.

- A very strong absorption at 3441 cm⁻¹ for rice TC and at 3442 cm⁻¹ for carbonized rice TC is observed and it can be attributed to O-H stretching vibration (PANDEY & PITMAN, 2003; OH et al., 2005; SINGH et al., 2014; SUN et al., 2005).

- The band at 2897 cm⁻¹ is due to the symmetric stretching vibration of C-H and is only present in the spectrum of rice TC (OH et al., 2005; SINGH et al., 2014; ZHANG et al., 2012).
3. Results & Discussion

The conjugated carbonyl group C=O stretching vibration, which is originated from lignin, is present in both spectra at 1641 and at 1645 cm\(^{-1}\) respectively (PANDEY & PITMAN, 2003; ZHANG et al., 2012).

At the spectrum of rice TC, in the fingerprint region, a number of bands are observed. Specifically, the bands at 1600, 1507 and 1426 cm\(^{-1}\) are assigned to the aromatic C=C stretching vibration from the aromatic rings of lignin (DORADO et al., 1999; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

The absorption bands at 1461 and 1371 cm\(^{-1}\) reflect the C-H symmetric and asymmetric deformation in lignin carbohydrates, respectively (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012).

The band at 1327 cm\(^{-1}\) is attributed to C-C and C-O skeletal vibrations (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012). Peaks between 1200 and 1000 cm\(^{-1}\) represent C-O stretching and deformation vibrations of cellulose, lignin and residual hemicellulose bands (SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

Specifically, the band at 1233 cm\(^{-1}\) is assigned to the C-O stretching vibration, which is indicative of hemicellulose-lignin linkage (OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014).

The peak at 1162 cm\(^{-1}\) is attributed to the C-O-C vibration in cellulose and hemicelluloses (KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SUN et al., 2005).

**Figure 38.** FT-IR spectra of rice husk TCs after delignification (Rice DL TC) and after carbonization (Rice DL carbonized TC) in the region 4000-400 cm\(^{-1}\).
### Table 19. Vibration bands of rice TC and carbonized rice TC.

<table>
<thead>
<tr>
<th>Band position (cm(^{-1}))</th>
<th>Rice TC</th>
<th>Carbonized rice TC</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3441</td>
<td>3442</td>
<td>(\nu\text{O-H stretching of silanol Si-OH groups on the silica surface})</td>
<td></td>
</tr>
<tr>
<td>2897</td>
<td>-</td>
<td>(\nu\text{C-H})</td>
<td></td>
</tr>
<tr>
<td>1641</td>
<td>1645</td>
<td>(\nu\text{C=O stretching vibration})</td>
<td></td>
</tr>
<tr>
<td>1600</td>
<td>-</td>
<td>(\nu\text{C=C stretching vibration from lignin aromatic rings})</td>
<td></td>
</tr>
<tr>
<td>1507</td>
<td>-</td>
<td>(\nu\text{C=C stretching vibration from lignin aromatic rings})</td>
<td></td>
</tr>
<tr>
<td>1461</td>
<td>-</td>
<td>(\nu\text{C-H symmetric deformation in lignin and carbohydrates})</td>
<td></td>
</tr>
<tr>
<td>1444</td>
<td>-</td>
<td>(\nu\text{C=C stretch from aromatic ring of lignin})</td>
<td></td>
</tr>
<tr>
<td>1426</td>
<td>-</td>
<td>(\nu\text{C=C stretching vibration from lignin aromatic rings})</td>
<td></td>
</tr>
<tr>
<td>1371</td>
<td>-</td>
<td>(\nu\text{C-H asymmetric deformation in lignin and carbohydrates})</td>
<td></td>
</tr>
<tr>
<td>1327</td>
<td>-</td>
<td>(\nu\text{C=C and C-O skeletal vibrations})</td>
<td></td>
</tr>
<tr>
<td>1233</td>
<td>-</td>
<td>(\nu\text{C-O indicative of hemicellulose-lignin linkage})</td>
<td></td>
</tr>
<tr>
<td>1162</td>
<td>-</td>
<td>(\nu\text{C-O-C vibration in cellulose and hemicellulose})</td>
<td></td>
</tr>
<tr>
<td>1119</td>
<td>-</td>
<td>(\nu\text{Aromatic skeletal and C-O})</td>
<td></td>
</tr>
<tr>
<td>1077</td>
<td>-</td>
<td>(\nu\text{C-O of carbohydrates})</td>
<td></td>
</tr>
<tr>
<td>1055</td>
<td>-</td>
<td>(\nu\text{C-O in cellulose and hemicellulose})</td>
<td></td>
</tr>
<tr>
<td>896</td>
<td>-</td>
<td>(\nu\text{Anomere C-groups, C1-H deformation ring valence vibration})</td>
<td></td>
</tr>
<tr>
<td>898</td>
<td>-</td>
<td>(\nu\text{C-1 group, (\beta)-glycosidic linkages between sugar units})</td>
<td></td>
</tr>
<tr>
<td>792</td>
<td>-</td>
<td>(\nu\text{Aromatic CH out-of-plain vibrations})</td>
<td></td>
</tr>
<tr>
<td>794</td>
<td>-</td>
<td>(\nu\text{Due to ring structure of SiO(_4)tetrahedra of silica})</td>
<td></td>
</tr>
<tr>
<td>663</td>
<td>-</td>
<td>(\nu\text{(\delta)-OH out-of-plane})</td>
<td></td>
</tr>
</tbody>
</table>

- The band at 1119 cm\(^{-1}\) reflects the aromatic skeletal and C-O stretching vibration and the band at 1055 cm\(^{-1}\) the C-O stretching vibration in cellulose and
hemicelluloses (ATTA et al., 2012; KACURAKOVA et al., 2000; KUHAD et al., 2010; PANDEY & PITMAN, 2003; SUN et al., 2005).

- Finally, the absorption peak at 898 cm\(^{-1}\) is indicative of the C-1 group or ring frequency and is characteristic of the β-glucosidic linkages between sugar units and the peak at 663 cm\(^{-1}\) is assigned to the C-OH out-of-plane bending mode (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

- At the spectrum of carbonized rice TC, in the fingerprint region, only few peaks are present. Particularly the bands at 1444 and 792 cm\(^{-1}\) are attributed to the aromatic C=C stretching vibrations and the aromatic CH out-of-plane vibrations, respectively and the band at 1077 cm\(^{-1}\) to the C-O stretching vibrations of carbohydrates (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SCHWANNINGER et al. 2004; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

### 3.5.2 FTIR analysis of mango TC

FT-IR analysis was also performed on mango TC after delignification and after carbonization. The FT-IR spectra in the region 4000-400 cm\(^{-1}\) are shown in Figure 39.

![Figure 39. FT-IR spectra of mango TC (Mango DL TC) and carbonized mango TC (Mango DL carbonized TC) in the region 4000-400 cm\(^{-1}\)](image)
### Table 20. Vibration bands for mango TC and carbonized mango TC.

<table>
<thead>
<tr>
<th>Band position (cm(^{-1}))</th>
<th>Mango TC</th>
<th>Mango carbonized TC</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3342</td>
<td>3427</td>
<td>νO-H</td>
<td></td>
</tr>
<tr>
<td>2899</td>
<td>-</td>
<td>ν(_{\text{C}-\text{H}})</td>
<td></td>
</tr>
<tr>
<td>1644</td>
<td>-</td>
<td>Conjugated carbonyl group C=O stretching vibration</td>
<td></td>
</tr>
<tr>
<td>1601</td>
<td>-</td>
<td>Aromatic C=C stretching vibration from lignin aromatic rings</td>
<td></td>
</tr>
<tr>
<td>1507</td>
<td>-</td>
<td>Aromatic C=C stretching vibration from lignin aromatic rings</td>
<td></td>
</tr>
<tr>
<td>1462</td>
<td>-</td>
<td>C-H symmetric deformation in lignin and carbohydrates</td>
<td></td>
</tr>
<tr>
<td>1426</td>
<td>1423</td>
<td>Aromatic C=C stretching vibration from lignin aromatic rings</td>
<td></td>
</tr>
<tr>
<td>1372</td>
<td>-</td>
<td>C-H asymmetric deformation in lignin and carbohydrates</td>
<td></td>
</tr>
<tr>
<td>1326</td>
<td>1317</td>
<td>C-C and C-O skeletal vibrations</td>
<td></td>
</tr>
<tr>
<td>1233</td>
<td>-</td>
<td>νC-O indicative of hemicellulose-lignin linkage</td>
<td></td>
</tr>
<tr>
<td>1162</td>
<td>-</td>
<td>C-O-C vibration in cellulose and hemicellulose</td>
<td></td>
</tr>
<tr>
<td>1119</td>
<td>-</td>
<td>Aromatic skeletal and νC-O</td>
<td></td>
</tr>
<tr>
<td>1050</td>
<td>1030</td>
<td>νC-O in cellulose and hemicellulose</td>
<td></td>
</tr>
<tr>
<td>898</td>
<td>875</td>
<td>C-1 group, β-glycosidic linkages between sugar units</td>
<td></td>
</tr>
<tr>
<td>663</td>
<td>-</td>
<td>δC-OH out-of-plane</td>
<td></td>
</tr>
</tbody>
</table>

The carbonized mango TC resulted to similar spectra but with quantitative differences of silicious compounds (smaller silicious content and more organic matter). The FT-IR spectra of mango TC and carbonized mango TC presented considerable changes in the whole spectral area. **Table 20** summarizes the vibration bands of the carbonized and non-carbonized mango TCs.
3. Results & Discussion

- A very strong absorption at 3342 cm\(^{-1}\) for mango TC and at 3427 cm\(^{-1}\) for carbonized mango TC is observed and it is attributed to O-H stretching vibration (PANDEY & PITMAN, 2003; OH et al., 2005; SINGH et al., 2014; SUN et al., 2005).
- The band at 2899 cm\(^{-1}\) is due to the symmetric stretching vibration of C-H and is only present in the spectrum of mango TC (OH et al., 2005; SINGH et al., 2014; ZHANG et al., 2012).
- The conjugated carbonyl group C=O stretching vibration, which is originated from lignin, is present only at the mango TC spectrum at 1644 cm\(^{-1}\) (PANDEY & PITMAN, 2003; ZHANG et al., 2012).
- At the spectrum of mango TC, in the fingerprint region, a number of bands are observed. Specifically, the bands at 1601, 1507 and 1426 cm\(^{-1}\) are assigned to the aromatic C=C stretching vibration from the aromatic rings of lignin (DORADO et al., 1999; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).
- The absorption bands at 1462 and 1372 cm\(^{-1}\) reflect the C-H symmetric and asymmetric deformation in lignin carbohydrates, respectively (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012).
- The band at 1326 cm\(^{-1}\) is attributed to C-C and C-O skeletal vibrations (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012).
- Peaks between 1200-1000 cm\(^{-1}\) represent C-O stretching and deformation vibrations of cellulose, lignin and residual hemicellulose bands (SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012). Specifically, the band at 1233 cm\(^{-1}\) is assigned to the C-O stretching vibration which is indicative of hemicellulose-lignin linkage (OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014).
- The peak at 1162 cm\(^{-1}\) is attributed to the C-O-C vibration in cellulose and hemicelluloses (KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SUN et al., 2005).
- The band at 1119 cm\(^{-1}\) reflects the aromatic skeletal and C-O stretching vibration and the band at 1050 cm\(^{-1}\) the C-O stretching vibration in cellulose and hemicelluloses (ATTA et al., 2012; KACURAKOVA et al., 2000; KUHAD et al., 2010; PANDEY & PITMAN, 2003; SUN et al., 2005).
- Finally, the absorption peak at 898 cm\(^{-1}\) is indicative of the C-1 group or ring frequency and is characteristic of the β-glycosidic linkages between sugar units and the peak at 663 cm\(^{-1}\) is assigned to the C-OH out-of-plane bending mode (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).
- At the spectrum of carbonized mango TC the bands at 3060 and 1793 cm\(^{-1}\) are attributed to the to the vibration mode of ν(=C-H) groups. In the fingerprint region, only few peaks are present. Particularly the band at 1619 cm\(^{-1}\) is assigned to the OH bending vibration of absorbed water, the bands at 1423 and 779 cm\(^{-1}\) are attributed to the aromatic C=C stretching vibrations and the aromatic CH out-of-plain vibrations respectively. The peak at 1317 cm\(^{-1}\) is characteristic of the C-C and C-O skeletal vibrations.
- Finally, the band at 1030 cm\(^{-1}\) reflects the C-O stretching vibration in cellulose and hemicellulose and the band at 875 cm\(^{-1}\) the C-1 group or ring frequency which is
characteristic of the β-glycosidic linkages between sugar units (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SCHWANNINGER et al. 2004; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

3.5.3 FTIR analysis of sal TC

FT-IR analysis was performed on sal TC after delignification and after carbonization. The FT-IR spectra in the region 4000-400 cm\(^{-1}\) are shown in Figure 40, and Table 21 summarizes the vibration bands of the carbonized and non-carbonized sal TCs.

![Figure 40](image)

**Figure 40.** FT-IR spectra of sal TC (Sal DL TC) and carbonized sal TC (Sal DL carbonized TC) in the region 4000-400 cm\(^{-1}\).

The FT-IR spectra of sal TC and carbonized sal TC presented considerable changes in the whole spectral area and especially in the fingerprint region.

- A very strong absorption at 3341 cm\(^{-1}\) for sal TC and at 3353 cm\(^{-1}\) for carbonized sal TC is observed and it is attributed to O-H stretching vibration (PANDEY & PITMAN, 2003; OH et al., 2005; SINGH et al., 2014; SUN et al., 2005).

- The band at 2916 cm\(^{-1}\) is due to the asymmetric stretching vibration of C-H and is only present in the spectrum of sal TC (OH et al., 2005; SINGH et al., 2014; ZHANG et al., 2012).
3. Results & Discussion

Table 21. Vibration bands of sal TC and carbonized sal TC.

<table>
<thead>
<tr>
<th>Band position (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal TC</td>
<td>Sal carbonized TC</td>
</tr>
<tr>
<td>3341</td>
<td>3353</td>
</tr>
<tr>
<td>2916</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>1795</td>
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<tr>
<td>1600</td>
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<td>-</td>
<td>1619</td>
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<td>1509</td>
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<td>1461</td>
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<td>1426</td>
<td>1421</td>
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<td>1374</td>
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<td>1329</td>
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<td>1268</td>
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<td>1118</td>
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<tr>
<td>1051</td>
<td>-</td>
</tr>
<tr>
<td>899</td>
<td>874</td>
</tr>
<tr>
<td>-</td>
<td>779</td>
</tr>
<tr>
<td>664</td>
<td>-</td>
</tr>
</tbody>
</table>

- At the spectrum of sal TC, in the fingerprint region, a number of bands are observed. Specifically, the bands at 1600, 1509 and 1426 cm\(^{-1}\) are assigned to the aromatic C=C stretching vibration from the aromatic rings of lignin (DORADO et al., 1999; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

- The absorption bands at 1461 and 1374 cm\(^{-1}\) reflect the C-H symmetric and asymmetric deformation in lignin carbohydrates, respectively (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012).

- The band at 1329 cm\(^{-1}\) is attributed to C-C and C-O skeletal vibrations (PANDEY & PITMAN, 2003; SUN et al., 2005; ZHANG et al., 2012).
3. Results & Discussion

- Peaks between 1200 and 1000 cm\(^{-1}\) represent C-O stretching and deformation vibrations of cellulose, lignin and residual hemicellulose bands (SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012). Specifically, the band at 1268 cm\(^{-1}\) is assigned to the C-O stretching vibration which is indicative of hemicellulose-lignin linkage (OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014).
- The peak at 1161 cm\(^{-1}\) is attributed to the C-O-C vibration in cellulose and hemicelluloses (KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SUN et al., 2005).
- The band at 1118 cm\(^{-1}\) reflects the aromatic skeletal and C-O stretching vibration and the band at 1051 cm\(^{-1}\) the C-O stretching vibration in cellulose and hemicelluloses (ATTA et al., 2012; KACURAKOVA et al., 2000; KUHAD et al., 2010; PANDEY & PITMAN, 2003; SUN et al., 2005).
- Finally, the absorption peak at 899 cm\(^{-1}\) is indicative of the C-1 group or ring frequency and is characteristic of the β-glycosidic linkages between sugar units and the peak at 664 cm\(^{-1}\) is assigned to the C-OH out-of-plane bending mode (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).
- At the spectrum of carbonized sal TC the band at 1795 cm\(^{-1}\) is attributed to the vibration mode of ν(=C-H) groups.
- In the fingerprint region, only few peaks are present. Particularly the band at 1619 cm\(^{-1}\) is assigned to the OH bending vibration of absorbed water, the bands at 1421 and 779 cm\(^{-1}\) are attributed to the aromatic C=C stretching vibrations and the aromatic CH out-of-plain vibrations, respectively. Finally the band at 874 cm\(^{-1}\) reflects the C-1 group or ring frequency which is characteristic of the β-glycosidic linkages between sugar units (GENG et al., 2003; KUHAD et al., 2010; OH et al., 2005; PANDEY & PITMAN, 2003; SINGH et al., 2014; SUN et al., 2005; ZHANG et al., 2012).

3.5.4 XRD analysis of rice TC

The XRD spectra of rice husk TC and carbonized rice husk TC are shown in Figure 41 (up). Three peaks were observed for all samples at 2θ=15.5°, 22.2° and 34.5°. These are characteristic of the crystal polymorphs of cellulose. The peaks at 2θ=15.5° corresponds to the (110) planes with corresponding crystal size of 66.3 Å, crystallographic peaks at 2θ=22.2° and 34.5° correspond to the (002) and (004) planes, respectively, with crystal size of 30.9 Å and 61.0 Å. Whereas comparing to the carbonized rice TC the crystallographic peaks at 2θ=15.5° were not present, although the crystallographic peaks at 2θ=26.5° showed the crystal size of 520.5 Å and crystallographic peaks at 2θ=29.3° showed the crystal size of 268 Å.
3. Results & Discussion

Figure 41. (up) XRD spectra of rice husk TC and carbonized rice husk TC; (down) XRD spectra of carbonized rice husk TC: red peaks show the presence of CaCO$_3$ and blue peaks show the presence of SiO$_2$.

The crystallinity index (CI) can be determined using the following equation (MWAIKAMBO et al., 2002):

$$CI = \frac{I_{(002)} - I_{(am)}}{I_{(002)}} \times 100$$
3. Results & Discussion

where $I_{(002)}$ is the peak intensity counter reading at $2\theta$ angle close to $22^\circ$ representing crystalline material and $I_{(am)}$ is the counter reading at at $2\theta$ angle close to $16^\circ$ representing amorphous material in the samples. Most of the peaks correspond to the presence of Si-H-K-Ca-O-H and mostly carbon in the carbonized rice TC. The crystallinity index obtained from XRD spectra for rice TC was found to be 52.3% and for carbonized rice TC 41.2%.

**Figure 41 (down)** shows the presence of calcium carbonate (CaCO$_3$), and silicon oxide (SiO$_2$) as a result of combustion after carbonization. In TC the increase of crystallinity is due to the removal of hemicellulose and lignin, which exist in the amorphous regions, leading to the realignment of cellulose molecules (LI et al., 2009).

### 3.5.5 XRD analysis of sal TC

The XRD spectra of sal TC and carbonized sal TC are shown in **Figure 42(up)**. Three peaks were observed similar to the previous samples at $2\theta=15.5^\circ$, $22.2^\circ$ and $34.5^\circ$. These are characteristic of the crystal polymorphs of cellulose as mentioned earlier. The peaks at $2\theta=15.5^\circ$ corresponds to the (110) planes with corresponding crystal size of 66.3 Å, crystallographic peaks at $2\theta=22.2^\circ$ and $34.5^\circ$ correspond to the (002) and (004) planes with crystal size of 30.9 Å and 61.0 Å, respectively. Whereas comparing to the carbonized sal TC the crystallographic peaks at $2\theta=15.5^\circ$ showed the crystal size of 249.3 Å, at $2\theta=22.2^\circ$ the crystal size of 445.4 Å, and at $2\theta=34.5^\circ$ the crystal size of 210.7 Å. This also concludes the decrease in the amorphous state and higher increase in the crystal state after the weight loss at 350ºC.

Therefore the crystallinity index obtained from XRD spectra was found to be 54.8 % for sal TC and 56.6 % for carbonized sal TC. **Figure 42(down)** shows the presence of CaCO$_3$, with traces of carbon, and SiO$_2$ as a result of cellular combustion after carbonization.
Figure 42. (up) XRD spectra of sal TC and carbonized sal TC; (down) XRD spectra of carbonized sal TC: red peaks show the presence of CaCO₃, blue peaks show the presence of SiO₂ in traces, and pink peaks show the presence of carbon in traces.
3. Results & Discussion

3.5.6 XRD analysis of mango TC

**Figure 43.** (up) Difference in the XRD spectra of mango TC and carbonized mango TC; (down) XRD spectra of carbonized mango TC: red peaks show the presence of CaCO$_3$, and blue peaks show the presence of SiO$_2$. 
3. Results & Discussion

The XRD spectra of TC from mango TC and carbonized mango TC are shown in Figure 43(up). Similar to the previous samples three peaks were observed at $2\theta = 15.5^\circ$, $22.2^\circ$ and $34.5^\circ$ that are the characteristic of the crystal polymorphs of cellulose. The crystallographic peaks at $2\theta = 15.5^\circ$ correspond to the (110) planes with corresponding crystal size of 45.5 Å, peaks at $2\theta = 22.2^\circ$ and $34.5^\circ$ correspond to the (002) and (004) planes with crystal size of 32.5 Å and 75.4 Å, respectively. Whereas comparing to the carbonized mango TC the crystallographic peaks at $2\theta = 15.8^\circ$ showed the crystal size of 204.3 Å, peaks at $2\theta = 26.5^\circ$ showed the crystal size of 584.6 Å and peaks at $2\theta = 29.3^\circ$ showed the crystal size of 284.7 Å. This also concludes the decrease in the amorphous state and higher increase in the crystalline state after the weight loss at 350°C.

Therefore the crystallinity index obtained from XRD spectra for mango TC and carbonized mango TC was found to be 58.8% and 74.4%, respectively. Figure 43(down) shows the presence of CaCO$_3$ and SiO$_2$ after carbonization that can be a result of combustion.

Chapter 6 - Application of Indian TCs as filters in cold pasteurization

3.6.1 General outcomes

The idea was to use Indian TCs for removal of microbial cells, having different dimensions, from liquid foods due to entrapment into the pores of TC. The experiments were performed using water and milk as model substrates. This type of cold pasteurization will have the advantages of negligible energy demand and unharmed nutritional value of the liquid foods compared to the conventional heat involving pasteurization processes.

Cold pasteurization of drinking water and milk was carried out in a system consisting of a sterile glass bioreactor packed with filters of 35 cm or 45 cm length, as shown in the schematic diagram of Figure 23 in the experimental part of the thesis and in the photos of Figure 45 below. The process was carried out under refrigeration temperature at 3-4°C. Both drinking water and milk were deliberately contaminated with S. cerevisiae or L. bulgaricus, respectively.

When the removal of microbial cell load was reduced below 80-90% the filter was regenerated with hot water in order to kill and wash away the entrapped cells. In the SEM photos of the filters after use (Figure 44) both S. cerevisiae (a) and L. bulgaricus (b) cells can be seen attached on the TC surface or entrapped in the TC tubes. The cell immobilization is probably done by hydrogen bonding between hydroxyl groups of the TC surface and the cell walls, or other weak forces, and by natural mechanical entrapment into the TC pores (BEKATOROU et al., 2014).
3. Results & Discussion

![Figure 44. SEM pictures showing entrapped *S. cerevisiae* (a) and *L. bulgaricus* (b) cells on the TC surface or in the TC tubes after cold pasteurization of drinking water.](image)

The results of the TC filters efficiency for microbial load removal from contaminated drinking water and skim/semi-skimmed milk are further analyzed below. It is clear that the longer the filter, the better the microbial load removal and operational stability, which was about 2-3 months depending upon the TC origin. During this time, the microbial load removal yield remained constant in the range of 98-100% for contaminated drinking water and 80-90% for skim milk and comparatively lower 70-80% for semi-skimmed milk, while only 3-4 regenerations of the filters were needed. From the results it was concluded that the filter should be bigger than 45 cm to acquire a satisfactory microbial load removal yield and continuous cold pasteurization duration. In industrial application, this could be achieved since the filter would be at least 1.5-2 m long. The regeneration of the filter can be done about once a week or even once a month depending upon the required microbial cell removal and the type of filter.

The reduction in the filter size not only lowers the microbial load removal and operational stability but also increases the number of necessary regenerations.

The origin of TC also plays a crucial role in the filter efficiency as it determines their surface characteristics (i.e. porosity, molecular groups available for hydrogen bonding, etc.) and mechanical strength (e.g. during pretreatment for delignification or for carbonization). Moreover, the uniformity in the particle size distribution of the wood sawdust is important for the proper bioreactor packing.

Finally, the presence of active species such as polyhydroxy polyphenol groups (e.g. of tannins and saponins) in wood sawdust can highly affect its biosorbent activity regarding uptake of cations and organic compounds such as dyes. Therefore, a main problem associated with such materials is the decoloration of the liquid foods during cold pasteurization. However, these properties may be useful when these materials are used for removal of organic pollutants like heavy metals and dyes from industrial effluents for safe deposition in the environment, given that they do not dissolve in the effluent leading to increase of its COD (POPA et al., 2010).
3.6.2 Cold pasteurization of drinking water

Water is not only important for sustaining life but is also essential for the survival of many organisms. About 70% of the whole human body by mass, excluding fat is composed of water. It is a crucial component for metabolic processes and serves as a solvent for many body solutes. Therefore, the safety and accessibility of drinking-water are major concerns throughout the world. Health risks may arise from the consumption of water contaminated with pathogenic microorganisms and toxic chemicals. Improving access to safe drinking-water and treatment of domestic wastewater can result in tangible improvements to health and water security problems, mainly in the third world countries where access to clean drinkable water is limited (WHO). Cold pasteurization using the Indian TCs was carried out using water contaminated with cells of different sizes, i.e. *S. cerevisiae* and *L. bulgaricus*.

The cold pasteurization process was carried out in the cylindrical column bioreactor packed with the different TCs (rice, mango and sal) (*Figure 45a*), at refrigeration temperature (4°C). The peristaltic pump used was set at the flow rate of 20 rpm/min controlling a flow of 2 mL per rotation through masterflex pipes of 2 mm diameter. The clarity of the effluent water was observed indicating that cell entrapment on the filter took place as also further confirmed by SEM (*Figure 45 b, c*).

*Figure 45. (a) The apparatus used for cold pasteurization; (b) The bioreactor packed with TC and clarity of the effluent; (c) Cells entrapped on TC.*
3.6.2.1 Cold pasteurization using rice husk TC filters

*Water contaminated with S. cerevisiae AXAZ-1*

The results of cold pasteurization of water contaminated with 0.2 and 0.5 g/L *S. cerevisiae* AXAZ-1 biomass using 35 cm rice TC filter are shown in Figure 46. For water contaminated with 0.2 g/L *S. cerevisiae* AXAZ-1, 85-99% of the microbial load was removed during the first 15 days (Figure 46 a). After decrease in microbial load removal down to 85% the first regeneration of the filter took place in order to wash away the entrapped cells. It was observed that the microbial load removal was increased to 98% after the first regeneration. This operation cycle was repeated and in the third week after the first regeneration a second regeneration took place as the microbial load removal again decreased to 85%. After the first and second regeneration was necessary, after which the microbial load removal increased up to 98-99%. However, after the second regeneration the filter efficiency decreased faster to 93-80% maybe due to deterioration of the filter or due to fouling by non-efficient cell removal during the regeneration processes.

The length of a filter and microbial concentration plays a crucial role in maintaining and controlling the microbial load removal. This was confirmed when the concentration of microbial load was increased to 0.5 g/L using the same 35 cm filter. It was concluded that increasing the microbial concentration results in necessity for more frequent regenerations, which result in faster decrease of the filter efficiency. The percentage of microbial load removal when increased cell concentration was used, with same filter length, was more or less similar (97-98%) as shown in Figure 46b.

![Figure 46. Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L *S. cerevisiae*, using 35 cm rice TC filter.](image_url)
3. Results & Discussion

Despite the relatively good results using the 35 cm TC filter, the higher filter length of 45 cm was also evaluated keeping the microbial concentration in water the same (Figure 47a, b).

For yeast concentration of 0.2 g/L the microbial load removal obtained was 95-99% with a first regeneration of the filter required at about 10 days and a second at 18 days of operation, since the target value for cells removal was 95% and higher. After the third regeneration at 25 days the filter efficiency started to drop resulting in microbial load removal lower than 90% (Figure 47a).

For yeast concentration of 0.5 g/L the efficiency of the filter for microbial load removal was similar to the previous (99-98%) but after the first and second filter regenerations the percentage decreased gradually to 75% (Figure 47b). To conclude, the increased microbial concentration in water affects the operational stability and filter efficiency, which is also affected on the origin of the TC and the number of regenerations.

The rice husk or hulls has a presence of high concentration of silica as well as the presence of meso and macro pores. Chemical activation of rice husk carbon lead to satisfactory purification of water from heavy metals and industrial textile dyes as compared with commercial carbon (TANNIN et al., 1988). Rice husks or hulls are significant high-volume agricultural wastes for the production of low-cost adsorbents (YOUSEFF et al., 1990). Yet scientific data are less available on the use of rice husk for microbial load removal as proposed in this investigation.

![Figure 47](image.png)

**Figure 47.** Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L *S. cerevisiae*, using 45 cm TC filter.
Water contaminated with L. bulgaricus

Similar experiments were also carried out for cold pasteurization of water contaminated with smaller bacterial cells of L. bulgaricus using rice husk TC filters 35 and 45 cm long (Figure 48 a,b). The cells concentration in water was the same in both cases (0.1 g/L). The 35 cm filter showed poor efficiency for microbial load removal (64-75%) requiring three regenerations. After the first regeneration at 6 days the microbial load removal was around 75% but then it decreased to 68% in less than a week when a second regeneration followed but with the same performance regarding microbial load removal (74%). After the third regeneration the filter efficiency was very weak and the microbial load removal did not present any significant improvements. Therefore, it can be concluded that the bacterial cells are much smaller and cannot be efficiently entrapped on the bigger TC filter pores.

For this reason, the same experiment was carried out with using a longer filter of 45 cm (Figure 48b). It was observed that with the increase of filter length the percentage of microbial load removal also increased to a certain extent 87-75 %, but compared to mango and sal TC filters it was lower. This can possibly be attributed to the lower mechanical strength of the rice husk TC and its more delicate structure which deteriorates after the repeated regenerations.

Figure 48. Cold pasteurization of drinking water contaminated with 0.1 g/L L. bulgaricus, using (a) 35 cm and (b) 45 cm rice TC filters.
3.6.2.2 Cold pasteurization using sal husk TC filters

Water contaminated with *S. cerevisiae*

Cold pasteurization of water with 0.2 g/L *S. cerevisiae* AXAZ-1 cells using 35 cm sal TC led to 90-99% microbial load removal during the first 16 days (Figure 49 a,b). After that decrease in microbial load removal to 90% was observed and the first filter regeneration took place. The microbial load removal was again increased to 100% and was maintained to about 95% for a week. Thus cold pasteurization of water using the 35 cm filter was effective for the given concentration (0.2 g/L) of yeast cells, and performance was better compared to rice and mango TCs.

Higher initial cell concentration in water was then applied (0.5 g/L) using the same filter length (35 cm) but it was concluded that increasing the microbial concentration results in slightly lower microbial load removal (95-80%) and more frequent required regenerations. However, compared to rice TC, the sal TC presented higher stability during 3-4 regenerations (Figure 49b).

Even though rice husk has a six fold higher BET surface than sal TC, its cell entrapment ability was lower. This may be attributed to the lower dimensions of the pores and tubes that cannot accommodate high numbers of big cells like yeast cells, as well as to the lower mechanical strength of husk compared to wood during the filter regenerations.

![Figure 49](image_url). Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L *S. cerevisiae*, using 35 cm sal TC filter.
3. Results & Discussion

The process was then scaled-up using a 45 cm sal TC filter (Figure 50a,b). The obtained microbial load removal was 100-95% with a first regeneration required at about 15 days and a second at 25 days in order to maintain a minimum microbial load removal percentage at 95%. Therefore, after the second regeneration in 25 days the filter efficiency was good (95-90%) (Figure 50a).

For water contaminated with 0.5 g/L yeast cells the microbial load removal was more or less similar to the previous (99-95%), with a slightly lower efficiency (90%) after the first and second regenerations (Figure 50b). Concluding, the sal TC has a better filtering ability achieving higher microbial load removal percentages and requiring fairly less regenerations compared to the rice husk TC.

![Graph](image)

**Figure 50.** Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L *S. cerevisiae*, using 45 cm sal TC filter.

**Water contaminated with *L. bulgaricus***

The same experimental work was carried out using water contaminated with the smaller *L. bulgaricus* cells. Sal TC filters of 35 and 45 cm length were used to filter water contaminated with 0.1 g/L cells. The 35 cm filter (Figure 51a) achieved a maximum microbial load removal of 84% and a minimum 74% and required three regenerations. After the first regeneration at 8 days the achieved removal was around 82% but then it slowly decreased to 75% in a week, followed by a second regeneration at 16 days and a third at 24 days. The removal was slightly higher during the first two days of each regeneration process but then it started to decrease to 75-72%. After the third regeneration the filter efficiency was weak and microbial
load removal did not show any significant improvements. Therefore, it is also concluded that the filter efficiency dropped due to not effective regeneration and fouling. TC deterioration was unlike to occur because the process conditions were very mild and wood is a more mechanically strong material compared to rice husk.

Finally, the same experiments were carried out using a filter of 45 cm length keeping the concentration of bacterial cells the same, i.e. 0.1 g/L. As in the previous cases, increasing the filter length increased the filter efficiency (microbial load removal 94-85%) (Figure 51b), compared to the rice TC and to the 35 cm sal TC filter. The filter efficiency slowly started to decrease every day until it reached the lower threshold value of 85%, then three regenerations took place and after the third regeneration, the efficiency of the filter was decreased significantly.

![Figure 51](image_url) Cold pasteurization of drinking water contaminated with 0.1 g/L _L. bulgaricus_, using (a) 35 cm and (b) 45 cm sal TC filters.

3.6.2.3 Cold pasteurization using mango husk TC filters

**Water contaminated with *S. cerevisiae***

The results of cold pasteurization of water containing 0.2 g/L yeast cells using the 35 cm mango TC filter are presented in Figure 52(a). The microbial load removal during the first 15 days was 90-99%, similar to that of the sal TC. When it was
decreased to 90% the first regeneration was done and the microbial load removal was increased up to 100% and it was maintained to 95% for about a week similarly to the sal TC. Therefore, the cold pasteurization of water with the 35 cm mango TC filter is effective for a microbial load of 0.2 g/L. The filter was more efficient compared to rice TC, with similar mechanical strength with sal TC but it required regeneration sooner than sal TC.

When higher concentration of microbial load was applied (0.5 g/L), it was concluded that more frequent regeneration was necessary, although compared to rice TC, mango TC could withstand 3-4 regenerations. The percentage of microbial load removal with the same 35 cm filter was slightly lower (90-85%) compared to sal TC as shown in Figure 52(b).

After the relatively satisfactory results for microbial load removal using the 35 cm filter the process was scaled-up employing a 45 cm filter and water containing 0.2 g/L and 0.5 g/L yeast cells. The achieved microbial load removal was 100-95%, and keeping the lower desired level to 95%, a first regeneration was needed at 12 days and a second at 24 days. After the second regeneration the filter efficiency was still good and the percentage of microbial load removal was around 95-90% (Figure 53a).

For initial cell concentration of 0.5 g/L, the filter efficiency was more or less similar to the previous (99-95%). After the first and second regeneration the microbial load removal remained about the same (considering a lower desired value of 90%), but after the third regeneration it started to decrease slowly below 80% (Figure 53b). Therefore, it can be concluded that mango TC is a good filtering support unlike rice TC, requiring less regenerations, but compared to sal TC it requires regeneration sooner.

Figure 52. Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L S. cerevisiae, using 35 cm mango TC filter.
Figure 53. Cold pasteurization of drinking water contaminated with (a) 0.2 g/L and (b) 0.5 g/L *S. cerevisiae*, using 45 cm mango TC filter.

**Water contaminated with L. bulgaricus**

Similar experiments were carried out regarding cold pasteurization of water contaminated with 0.1 g/L *L. bulgaricus* using the 35 and 45 cm mango TC filters. The results for the 35 cm filter are presented in Figure 54(a) showing that the maximum microbial load removal was 82% and the minimum 72% after which three regenerations followed. After the first regeneration at 7 days, the microbial load removal was 81% but then it slowly decreased to 70% within a week. Then a second regeneration followed at 16 days and a third at 24 days. The microbial load removal was slightly higher during the first two days after regeneration but then it started to decrease to 81-70% and 70-68% after the second and third regeneration, respectively. After the third regeneration the filter efficiency was too weak like in the previous cases of rice and sal TCs.

A 45 cm filter was also used leading to higher microbial load removal (92-85%) as compared to rice TC (Figure 54b) and the 35 cm mango TC filter. The microbial load removal efficiency slowly started to decrease gradually until it reached the lower desired value of 85%, after which regenerations followed. After the third regeneration the efficiency of the filter decreased significantly.
3. Results & Discussion

3.6.3 Cold pasteurization of milk

Skim or skimmed milk is milk from which fat has been removed. In some packaged milk covers it is labeled as “fat free” milk. FDA approves products to be labeled as “skim milk” when they have less than 0.5% fat, whereas low fat or semi-skimmed milk contains 1-2% fat. The results of cold pasteurization of water showed that the 45 cm filters performed better; therefore this length was used for the cold pasteurization experiments of milk. Likewise, because milk usually contains lactic acid bacteria, the experiments were carried out using milk contaminated with *L. bulgaricus* cells.

3.6.3.1 Cold pasteurization of skimmed milk

Skimmed milk containing 0.1 g/L of *L. bulgaricus* was filtered using the 45 cm sal TC filter (Figure 55a) leading to 96-90% microbial load removal during the first weeks. When this percentage was reduced to 89% the first filter regeneration took place leading to 94-89% removal. The second week after the first regeneration the cold pasteurization cycle was repeated until the microbial load removal decreased to

![Graph showing microbial removal over time](image-url)
87%. The filter efficiency was slightly improved just after each of the following two regenerations but then it was stably decreased as observed in the case of water. When higher cell concentration was used (0.2 g/L) it was shown that the removal was only 70-80% (Figure 55b).

![Figure 56](image)

**Figure 55.** Cold pasteurization of skimmed milk contaminated with (a) 0.1 g/L and (b) 0.2 g/L *L. bulgaricus*, using 45 cm sal TC filter.

In the case of skimmed milk (0.1 g/L of *L. bulgaricus*) pasteurization using the 45 cm mango TC filter (Figure 56a), 81-91% of removal was achieved during the first week, which is slightly lower than that of the sal TC. Regeneration took place when removal dropped to 80%, leading to a significant rise at 90%. Then again the percentage gradually decreased during the second week of continuous cold pasteurization until 79% on day 14. Then the second regeneration was done, and so on. After the third regeneration the microbial load removal was not more than 84%, again with decreasing trend day by day, until no further regenerations would result in any improvements.

The process was again repeated with skimmed milk containing 0.2 g/L cells, which reduced the efficiency of the process to 67-74%. Even after the following three regenerations the microbial load removal did not exceed the desired threshold of 70% with a minimum at 57% (Figure 56b). It was also concluded that the efficiency of the TC was minimized after the fourth and fifth washings indicating deterioration or fouling of the filter.
3. Results & Discussion

Figure 56. Cold pasteurization of skimmed milk contaminated with (a) 0.1 g/L and (b) 0.2 g/L *L. bulgaricus*, using 45 cm mango TC filter.

3.6.3.2 Cold pasteurization of semi-skimmed milk

Cold pasteurization of semi-skimmed milk containing 0.1 g/L of *L. bulgaricus* using the 45 cm sal TC filter (**Figure 57a**) led to 77–67% microbial load removal during the first week. The first regeneration was then carried out leading to a slight increase of cell removal to 74–69%. The second week after the first regeneration the cycle was again repeated and when the microbial load removal decreased to about 63% the second and third regenerations took place leading to slight improvements but not better than the level obtained in the first week. Thus, it can be concluded that for semi-skimmed milk not only the filter length and microbial cell concentration is important but also the fat content which may have inhibited the entrapment of cells due to clogging on the surface of TC or other interactions.

The same experiments were carried out using semi-skimmed milk containing 0.2 g/L cells. It was shown that only ~57–48% microbial load removal was possible (**Figure 57b**).
Cold pasteurization of semi-skimmed milk was also carried out with the 45 cm mango TC filter, which had similar filtering efficiency and morphological characteristics with sal TC as described earlier. Initially 65-72% of microbial load removal was observed during the first week for 0.1 g/L of \textit{L. bulgaricus} concentration, which is slightly lower than that obtained by sal TC (Figure 58a). After decrease of microbial load removal down to 65% the first regeneration was done, which led to a significant increase of removal to 71%; however this percentage slowly started to decrease to 64% in less than a week of continuous operation. The second regeneration was then carried out as in the case of sal TC. After the second and third regeneration it was observed that the microbial load removal increased but at lower levels than the first week of operation and the first regeneration.

The process was again repeated with higher microbial concentration (0.2 g/L) leading to lower percentage of microbial load removal (47-52%) without any significant improvements after five regenerations (up to 40% removal achieved during 20 days of operation) (Figure 58b).
3. Results & Discussion

3.6.3.3 Sensory evaluations of pasteurized milk products

Sensory evaluations of the milk products were performed before and after the cold pasteurization to evaluate if the process caused any textural or sensory changes. After the cold pasteurization the presence of lactic acid was detected indicating that LAF occurred due to the presence of high numbers of *L. bulgaricus*, which is known to be an acidophilic species. Probably lower temperatures during the cold pasteurization process could have reduced the fermentative activity of *L. bulgaricus*.

3.6.3.4 Conclusions

The results of cold pasteurization of water and milk clearly indicated that since water has lower viscosity than milk it allows the filter to more efficiently remove the microbial load. Likewise, the morphological characteristics of the TC materials (number and size of tubes, surface area, average pore size) also seem to play a substantial role on filter efficiency during the cold pasteurization process as well as the fermentation performance of cells immobilized on the TCs as discussed in the following chapters.

Regarding cold pasteurization of milk, the efficiency of the TC filters was better in skimmed milk, because the fat content of the semi-skimmed milk caused faster filter...
fouling. Furthermore, the combination of lower temperature pasteurization (below 4°C) could also be examined to avoid deterioration of milk quality due to undesired milk fermentation by the entrapped bacteria on the filter.

Comparing the Indian origin rice, mango, and sal TCs employed in the cold pasteurization experiments, it can be concluded that rice husk TC was the less suitable material due to its more delicate character (lower mechanical strength) during the continuous operation and repeated regenerations, and faster fouling especially in the case of viscous products such as milk. In all cases, it was found that the higher the filter length the better the microbial load removal efficiency.

Comparing the wood sawdusts, both sal and mango TCs proved to be more advantageous compared to rice TC for the development nano/micro filters for liquid food cold pasteurization processes. They presented higher mechanical strength and better microbial load removal efficiency. However, rice husk TC was also suitable for cold pasteurization of low viscosity fluids such as water. Therefore, these materials can be proposed for cold pasteurization of drinking water in rural areas where lignocellulosic biomass is abundant such as India.

These materials could also be utilized for microfiltration in waste water treatment after the processes of coagulation and sedimentation, as well as for industrial and domestic filtering purposes. The advantages of such materials for practical applications are the high microbial load removal efficiency in water, their availability and low cost and the simple methodology required.

Chapter 7 - Fermentations

3.7.1 Promotion of lactic acid fermentation by Indian origin TCs

The promotion of lactic acid fermentation (LAF from this point on in this section of the thesis) was studied using cheese whey and lactose synthetic media as substrates and the thermophilic, homofermentative *L. bulgaricus* immobilized on the different Indian TCs as biocatalysts. The use of mango, sal and rice husk TCs, especially of Indian origin, for LAF by *L. bulgaricus* has not been reported. Thus, since they are abundant, renewable and cheap materials they could be used for the production of immobilized cell biocatalysts for ethanol and lactic acid production useful for food bioprocessing.

The results indicated that the increased fermentation efficiency of *L. bulgaricus* in the presence of TC can be attributed (apart from the effect of immobilization itself on cell performance) to catalysis by the TC material with in turn is affect by its porosity: higher surfaces area contributed to shorter fermentation time.

Additionally to the TCs as cell carriers, TC composites with alginate and PLA were also evaluated regarding their fermentation activity. The composites were prepared
in order to stabilize the alginate beads, which are commonly used in immobilized cell studies but are easily disrupted.

![Image of composite beads](image1.png)

**Figure 59.** Mango TC/alginate/PLA (a) and mango TC/PLA (b) composite beads with immobilized *L. bulgaricus*; (c) TC in synthetic medium fermentation d) TC Alginate composite in synthetic media fermentation.

The TC/alginate/PLA and TC/PLA composites with immobilized *L. bulgaricus* are shown in **Figure 59a,b.** The composites where found to be suitable for 3-4 repeated fermentation batches without any disruption and with steady fermentation rates. The PLA used in the composites, and therefore the proposed biocatalysts, were of good grade purity. They are cheap and easy to prepare, and they are attractive for bioprocess development based on immobilized cells. Such composite biocatalysts may be used for the co-immobilization of different microorganisms or enzymes (in separate layers of the biocatalysts), to efficiently conduct different types of
fermentation in the same bioreactor, avoiding inhibition problems of chemical or biological competition between cells of different microorganisms (SERVETAS et al., 2013). Figure 60 shows SEM images of immobilized L. bulgaricus cells on mango, rice, and sal TC effectively supporting the lactic acid repeated batch fermentations both in cheese whey and lactose medium.

The suitability of the TC and the TC/alginate/PLA composite biocatalysts for LAF was evaluated in lactose media and cheese whey. The fermentation was carried out at 37°C and the °Be hydrometer density (proportional to sugar concentration) versus time was recorded in order to compare the fermentation ability of each biocatalyst. Figure 61-Figure 63 show the repeated fermentation batches using the different biocatalysts in cheese whey.

From Figure 61 it can be seen that the decrease in sugar density with mango, sal, and rice husk TC in LAF of cheese whey was faster compared with free cells. Sal TC presented faster decrease in the sugar density compared to other TCs.
3. Results & Discussion

Figure 61. Kinetics of LAF of cheese whey using *L. bulgaricus* cells immobilized on the different TCs of Indian origin.

Figure 62. Kinetics of LAF of cheese whey using *L. bulgaricus* cells immobilized on the different TC/alginate/PLA composites.
In the same context, Figure 62 shows that LAF kinetics of cheese whey using the mango, sal, and rice TC/alginate/PLA composites to be much faster compared with free cells. Also as compared with the previous kinetics from Figure 61 it shows that faster fermentation rates were obtained by the composites than by the plain TCs. The improved fermentation efficiency may be attributed to the better protection of the cells entrapped in the composites from sear forces and chemical inhibitors (e.g. lactic acid, low pH, etc.). Here the mango TC composite exhibited the best fermentation activity as compared with rice and sal TCs. This may be due to a better adhesion of cells and/or better firmness and stability of the composite. Generally, all composites performed better as compared with free cells.

In Figure 63, a comparison between the composite and plain mango TC is shown. A faster fermentation rate can be observed in the TC/alginate/PLA composite kinetics. It is clear that the composite biocatalyst is more active and causes a substantially higher promotion of LAF compared to the plain TC. This promotional effect on fermentation observed when cells immobilized on delignified cellulosic materials are used, can be in general explained by a sugar (glucose, maltose, lactose, etc.) pump theory as it has been suggested by other researchers (KOUTINAS et al., 2012; GANATSIOS et al., 2014).

![Figure 63](image)

**Figure 63.** Kinetics of LAF of cheese whey using *L. bulgaricus* cells immobilized on mango TC/alginate/PLA composite and plain mango TC.
3. Results & Discussion

3.7.1.1 Summary of results and quality of the LAF products

Cell immobilization on the mango, sal and rice TCs and their Ca-alginate/PLA composites was proved by SEM and was further validated by the ability of the biocatalysts to perform LAF of whey (50 g lactose/L) and synthetic lactose media (20 g lactose/L). The results are summarized in Table 22.

<table>
<thead>
<tr>
<th></th>
<th>Lactic acid (g/L)</th>
<th>Residual sugar (g/L)</th>
<th>Conversion (%)</th>
<th>Lactic acid yield (g/g)</th>
<th>Lactic acid productivity (g/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cheese whey</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mango TC/alginate/PLA composite</td>
<td>23.1</td>
<td>9.1</td>
<td>81.8</td>
<td>0.462</td>
<td>5.78</td>
</tr>
<tr>
<td>Rice husk TC/alginate/PLA composite</td>
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<td>9.8</td>
<td>80.4</td>
<td>0.458</td>
<td>5.73</td>
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<td>Sal TC/alginate/PLA composite</td>
<td>22.1</td>
<td>10.1</td>
<td>79.8</td>
<td>0.442</td>
<td>5.53</td>
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<td>Mango TC</td>
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<td>4.5</td>
<td>91</td>
<td>0.542</td>
<td>6.78</td>
</tr>
<tr>
<td>Rice husk TC</td>
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<td>8.6</td>
<td>82.8</td>
<td>0.462</td>
<td>5.78</td>
</tr>
<tr>
<td>Sal TC</td>
<td>23.8</td>
<td>9.1</td>
<td>81.8</td>
<td>0.476</td>
<td>5.95</td>
</tr>
<tr>
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<td>10.4</td>
<td>79.2</td>
<td>0.392</td>
<td>4.90</td>
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<tr>
<td><strong>Lactose</strong></td>
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<td></td>
</tr>
<tr>
<td>Mango TC/alginate/PLA composite</td>
<td>28.1</td>
<td>4.8</td>
<td>90.4</td>
<td>0.562</td>
<td>7.03</td>
</tr>
<tr>
<td>Rice husk TC/alginate/PLA composite</td>
<td>26.4</td>
<td>5.1</td>
<td>89.8</td>
<td>0.528</td>
<td>6.60</td>
</tr>
<tr>
<td>Sal TC/alginate/PLA composite</td>
<td>24.1</td>
<td>5.9</td>
<td>88.2</td>
<td>0.482</td>
<td>6.03</td>
</tr>
<tr>
<td>Mango TC</td>
<td>30.2</td>
<td>3.9</td>
<td>92.2</td>
<td>0.604</td>
<td>7.55</td>
</tr>
<tr>
<td>Rice husk TC</td>
<td>27.7</td>
<td>3.7</td>
<td>92.6</td>
<td>0.554</td>
<td>6.93</td>
</tr>
<tr>
<td>Sal TC</td>
<td>28.5</td>
<td>4.9</td>
<td>90.2</td>
<td>0.57</td>
<td>7.13</td>
</tr>
<tr>
<td>Free Cells</td>
<td>20.6</td>
<td>8.4</td>
<td>83.2</td>
<td>0.412</td>
<td>5.15</td>
</tr>
</tbody>
</table>

The conversion of lactose was higher than 80% in all cases, but both free and immobilized cells performed better in the lactose media than in cheese whey, leading to higher conversion rates and higher lactic acid concentrations, yields (g lactic acid/g sugar utilized) and productivities (g lactic acid per L total working volume and
This may be due to lower substrate inhibition, since the lactose concentration in the synthetic media was lower, or more possibly to the complete medium composition compared to cheese whey which was not enriched by any nutrient addition. Lactic acid yields and productivities were significantly higher in the case of the biocatalysts compared to free cells (at the P < 0.05 level).

Among the different immobilized systems used, the best results were obtained in the case of both plain and composite mango TC biocatalysts. However, the differences observed between all the immobilized biocatalysts were not statistically significant (P < 0.05). The better performance of the immobilized *L. bulgaricus* cells may be attributed to the protective effects of the immobilization matrix against stress, shearing forces and inhibitory effects of substrates, media components and fermentation products, as well as to altered physiology and metabolic activity in general due to immobilization as discussed earlier.

**Figure 64** shows the comparison of cheese whey and synthetic medium fermentation using the composite and plain mango TC. It can be observed that the composite is the most effective biocatalyst for whey fermentation.

![Figure 64](image)

**Figure 64.** Kinetics of LAF of cheese whey and synthetic medium using *L. bulgaricus* immobilized on mango TC/alginate composite and plain mango TC.

The fermentation time using the composite biocatalyst was about 20 hours. Therefore, TC is a weaker promoter in LAF compared to alcoholic fermentation as observed in previous studies due to the biochemical nature of these two metabolic pathways, the higher fermentative activity of yeasts, and the higher inhibition by the end products of LAF.
3. Results & Discussion

The results of the semi-quantitative SPME-GC-MS analysis of the headspace volatile compounds of the products of lactose and whey fermentation by encapsulated *L. bulgaricus* cells on the different TC/Ca-alginate/PLA composites are shown in Table 23. The analysis was performed to evaluate the effect of the immobilized biocatalysts on the formation of volatile by-products that may affect the quality of a fermented product destined for food production. Alcohols, esters, carbonyl compounds and organic acids that can potentially influence the flavor of a fermented food product were detected.

In the case of cheese whey, the fermentation products of the biocatalysts presented a more complex profile and higher amounts of volatile by-products compared to free cells as previously shown in alcoholic fermentation products (MALLOUCHOS et al., 2003; SERVETAS et al., 2013; PLESSAS et al., 2007). Moreover, higher amounts of volatile compounds were found in the fermented whey samples compared to the synthetic lactose media, a number of which may be derived from the substrate (KATECHAKI et al., 2010) and the rest are fermentation by-products.

The major volatiles produced were organic acids and alcohols. Esters were also produced, which can affect a fermented product flavor as they usually have low odor threshold values and pleasant flowery and fruity aromas (MALLOUCHOS et al., 2003). The other groups of compounds generally contribute to the complexity of the aroma, except specific compounds such as phenylethanol, benzaldehyde, etc., with characteristic odors and low threshold values. However, that increase in total volatiles is mainly due to high increase of concentrations of organic acids. Esters were also increased as compared with free cells in whey fermentation for mango composite. Alcohols were also substantially increased as compared with free cells in all cases were studied.

Taking into account the results of the effect of Indian TCs and their composites on the rate of whey fermentation one can conclude that they can play an important role in lactic acid production, which is a high value chemical used in foods, pharmaceuticals, cosmetics and for production of PLA, a biodegradable polymer. The results indicate that the lower lactose concentration of 20 g/L allows high conversion up to 92% and increased productivity (7.55 g/L/d). These result seems low because it was calculated taking into account the total fermentation time during which the reduction of lactose concentration was negligible. Considering that the time to ferment lactose was practically 20 hours, the productivity is calculated as 27.6 g/L/d, which is considerable. Furthermore the production of adequate amounts of volatiles by the Indian TCs that can contribute to flavor enhancement in fermented foods opens the way for their use in food production industry.
Table 23. Volatile compounds (mL/L) identified in the fermentation products of cheese whey and lactose media at 37°C using free and immobilized *L. bulgaricus* cells on mango, rice and sal TC/Ca-alginate/PLA composites.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TC/alginate/PLA composite</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>KI</td>
<td>Free cells</td>
<td>Whey</td>
<td>Mango</td>
<td>Rice</td>
<td>Sal</td>
<td>Mango</td>
<td>Rice</td>
<td>Sal</td>
<td>Mango</td>
<td>Rice</td>
</tr>
<tr>
<td><strong>Esters</strong></td>
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<td></td>
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</tr>
<tr>
<td>Ethyl acetate</td>
<td>864</td>
<td>1.04</td>
<td>0.50</td>
<td>0.94</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>1394</td>
<td>0.42</td>
<td>0.06</td>
<td>1.16</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Methyl nonanoate</td>
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<td></td>
<td>2.68</td>
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<td>Methyl benzoate</td>
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<tr>
<td>Methyl nonadecanoate</td>
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<tr>
<td>2-phenylethyl acetate</td>
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<td></td>
<td></td>
<td>0.39</td>
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<td></td>
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<tr>
<td>Butyl octanoate</td>
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<tr>
<td><strong>Total Esters</strong></td>
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<td>0.34</td>
<td>2.46</td>
<td>0.50</td>
<td>1.19</td>
<td>0</td>
<td>3.98</td>
<td>1.35</td>
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<td><strong>Alcohols</strong></td>
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<td>Ethanol</td>
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<td>Isopentyl alcohol</td>
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<td>1-Hexanol</td>
<td>1445</td>
<td>0.84</td>
<td>0.36</td>
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<td>2-Nonanol</td>
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<td>4.47</td>
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<td>Phenylethyl Alcohol</td>
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<td>3.73</td>
<td>96.75</td>
<td>93.11</td>
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<tr>
<td>Decanoic acid</td>
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<td>45.50</td>
<td>42.74</td>
<td>36.84</td>
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<tr>
<td><strong>Total Organic Acids</strong></td>
<td></td>
<td>45.50</td>
<td>98.90</td>
<td>157.93</td>
<td>146.15</td>
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<td>13.73</td>
<td>25.81</td>
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<tr>
<td><strong>Carbonyl compounds</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hexanal</td>
<td>1067</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Heptanone</td>
<td>1172</td>
<td>0.25</td>
<td>0.41</td>
<td>1.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2-Decanone</td>
<td>1344</td>
<td></td>
<td></td>
<td>5.66</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1477</td>
<td>0.39</td>
<td>1.34</td>
<td>0.72</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2-Undecanone</td>
<td>1542</td>
<td>0.44</td>
<td>0.17</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Total carbonyls</strong></td>
<td></td>
<td>0.39</td>
<td>1.34</td>
<td>1.16</td>
<td>0.67</td>
<td>0.41</td>
<td>0</td>
<td>8.45</td>
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<tr>
<td><strong>Total compounds</strong></td>
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<td>169.91</td>
<td>157.86</td>
<td>33.48</td>
<td>28.17</td>
<td>61.63</td>
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<td></td>
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<tr>
<td><strong>Total no. of compounds</strong></td>
<td></td>
<td>4</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. Results & Discussion

3.7.2 Promotion of alcoholic fermentation by Indian origin TCs

Alcoholic fermentation of glucose synthetic media and grape must using the alcohol resistant and cryotolerant yeast *S. cerevisiae* AXAZ-1, isolated from a Greek vineyard (ARGIRIOU et al., 1996) was immobilized on the TCs of Indian origin and was studied in order to evaluate the fermentation activity of the produced biocatalysts. The use of the proposed biocatalysts in alcoholic fermentation processes has not been reported. The fermentation was carried out at low temperatures of 15°C simultaneously. Immobilization took place by mixing the delignified cellulosic support with a liquid culture of *S. cerevisiae* AXAZ-1 and the system was left to ferment for 8-12 h. The immobilization was confirmed by SEM (Figure 65 a,b,c), showing yeast cells attached on the surface of TCs. An enumeration of the immobilized cells by microbiological analysis showed that more than \(7.1 \times 10^7\) cells/g of TC are immobilized. In addition the immobilization was proved by using the biocatalysts for repeated batch fermentations of glucose synthetic medium and grape must at 15°C.

![SEM image of *S. cerevisiae* cells immobilized on (a) mango (b) sal and (c) rice TC.](image)

The sugar density (°Bé) against time was recorded to compare the fermentation capability and operational stability of the biocatalyst. Below are the graphs showing the repeated alcoholic batch fermentations of each biocatalyst in 12% glucose synthetic medium at 15°C. The observed increased fermentation efficiency of the immobilized biocatalysts can be attributed to the catalytic effect of the TC carrier.
Higher lactic acid yields (g/g sugar utilized) and productivities (g/L/d) and higher amounts of volatile by-products were achieved, while no significant differences were observed on the performance of the different immobilized biocatalysts.

**Figure 66** shows the kinetics of the repeated batch alcoholic fermentations using the mango TC biocatalyst. It is clear that the first fermentation batch showed a slower but consistent fermentation rate as compared to other 2-4 repeated batches where the fermentation time was fairly lower.

![Kinetics of the alcoholic fermentation of glucose using mango TC at 15 °C.](image)

**Figure 66.** Kinetics of the alcoholic fermentation of glucose using mango TC at 15 °C.

Sal TC showed a faster rate of glucose fermentation for batch 1 (**Figure 67**), whereas compared to other repeated batches 2-4 the fermentation rate was slower. The delay of fermentation during the first batch in all cases is due to the time needed for the cells to adapt to the fermentation environment as well as to microbial cell growth that takes place during fermentation and therefore increased cells densities in the bioreactor from batch to batch.
3. Results & Discussion

Figure 67. Kinetics of the alcoholic fermentation of glucose using sal TC at 15°C.

Figure 68. Kinetics of the alcoholic fermentation of glucose using rice TC at 15°C.

Figure 68 shows similar and faster decrease in sugar density for batch 1 using the sal TC, whereas compared to batches 2-4 the fermentation rate was slightly slower.
However, the duration of fermentation time was more or less the same for all batches, which is a significant difference in comparison to the other TC biocatalysts. The results presented above show a differentiation in the fermentation times between the successive fermentation batches. This is made clearer by the data presented in Table 24.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Mango TC</th>
<th>Sal TC</th>
<th>Rice TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{st}</td>
<td>104</td>
<td>112</td>
<td>107</td>
</tr>
<tr>
<td>4\textsuperscript{th}</td>
<td>92</td>
<td>147</td>
<td>122</td>
</tr>
</tbody>
</table>

Therefore, it is proved that mango TC is the most effective in comparison with sal and rice, showing a progressive reduction of the fermentation time from the 1\textsuperscript{st} to the 4\textsuperscript{th} repeated batch. Likewise, sal and rice TCs resulted respectively to a 60\% and 30\% increase of fermentation time as compared with mango. These results prove that the origin of TC has a substantial effect on alcoholic fermentation efficiency.

Based on these findings, experiments to compare Indian TC with Greek TC were carried out and the kinetics of the 1\textsuperscript{st} batch are presented in Figure 69, which illustrates that the Greek origin TC ferments faster during the first hours of fermentation but slows down when the ethanol concentration increases. The opposite was observed for the Indian TCs which fermented with stable rate until all sugar was utilized. The fermentation time for the Greek origin TC was near to that of mango TC for the 1\textsuperscript{st} batch of fermentation.
Figure 69. Comparative glucose alcoholic fermentation kinetics (1st batch) for Indian and Greek TCs at 15°C.

Figure 70 a,b shows the comparative kinetics for the 2nd and 3rd batches. The fermentation kinetics for mango TC and Greek TC were significantly improved compared to the 1st batch, while sal and rice activity was reduced. Likewise, the fermentation times for mango and Greek TC were the same. The results indicating good efficiency of the Indian TCs at high ethanol concentration can be further utilized and scientifically exploited to obtain higher ethanol concentrations that are necessary for the reduction of the energy demands in the alcohol production units.
Figure 70. Comparative glucose alcoholic fermentation kinetics of 2nd batch (up) and 3rd batch (down) for Indian and Greek TCs at 15°C.
3.7.2.1 Promotion of alcoholic fermentation by composite TC biocatalysts

Immobilized *S. cerevisiae* cells in TC/alginate/PLA composite were also used for alcoholic fermentation as in the case of LAF. The composites were found to be suitable for 3-4 repeated batches without any indication of cell disruption and with steady fermentation performance. The results also indicated a catalytic promotional effect of TC on fermentation rates, variation in their pore size and pore diameters to have more surface area for faster and repeated batch for alcoholic fermentations. Fermentation of glucose and grape must using the composite TC biocatalysts was carried out at 15°C. The sugar density against time was recorded to compare the fermentation efficiency of each biocatalyst.

The 1st fermentation batch for all the TC composite biocatalysts (mango, sal & rice) was faster compared to the plain TC biocatalysts. Figure 71 below shows the kinetics of repeated batch fermentations using the mango TC composite biocatalyst in grape must. A significant difference was observed in the 4th and 6th batches, where the highest fermentation rates were observed, while the fermentation efficiency was after the 8th repeated batch.

![Figure 71. Kinetics of alcoholic fermentation for grape must, using the mango TC/alginate/PLA composite biocatalyst at 15°C.](image)

**Figure 71.** Kinetics of alcoholic fermentation for grape must, using the mango TC/alginate/PLA composite biocatalyst at 15°C.

**Figure 72** below shows the repeated batch fermentations for rice TC composite biocatalyst. Similarly as in the case of mango composite biocatalyst, the highest
fermentation rates were observed in the 4th and 6th batches, and fermentation efficiency was reduced after the 8th batch.

**Figure 72.** Kinetics of alcoholic fermentation for grape must, using the rice TC/alginate/PLA composite biocatalyst at 15°C.

The same fermentation behavior was observed for the sal TC composite biocatalyst (Figure 73). The fermentation rate was slower in the 8th batch with higher duration of fermentation than both mango and rice composite biocatalysts. However, the composite biocatalysts were efficient up to the 8th batch, stably consuming almost all sugar, compared to the plain TC biocatalysts, which were efficient up to the 4th fermentation batch.
Figure 73. Kinetics of alcoholic fermentation of grape must using sal TC/alginate/PLA composite biocatalyst at 15°C.

Figure 74. Comparative kinetics of glucose alcoholic fermentation (1st Batch) at 15°C by the Indian TC/alginate/PLA composite biocatalysts

Therefore, it is proved again that among the TC/alginate/PLA composite biocatalysts, mango biocatalyst was the most effective in comparison with rice and sal, which resulted respectively to a 70% and 30% increase of fermentation time as compared
with mango. These results prove that the origin of TC used in the composite biocatalysts affects their alcoholic fermentation efficiency and economic output. Based on these findings, experiments to compare the different Indian TC composite biocatalysts were performed, and the kinetics of the 1st batch are presented in Figure 74. This figure illustrates that the mango biocatalyst promotes a substantial increase on the rate of fermentation compared to rice and sal, without reduction of fermentation rate at high alcohol concentration.

The results presented above show a differentiation on the fermentation performance of the different composite biocatalysts, which is more clearly presented by the data in Table 25.

**Table 25.** Comparison of fermentation kinetics of the Indian origin TC/alginate/PLA composite biocatalysts at 15°C.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Mango TC Composite</th>
<th>Rice TC Composite</th>
<th>Sal TC Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>85</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>4th</td>
<td>50</td>
<td>85</td>
<td>135</td>
</tr>
<tr>
<td>6th</td>
<td>50</td>
<td>90</td>
<td>136</td>
</tr>
<tr>
<td>8th</td>
<td>124</td>
<td>140</td>
<td>140</td>
</tr>
</tbody>
</table>

**Table 26** provides kinetic parameters of glucose and grape must fermentations using the Indian TC/alginate/PLA composite biocatalysts. The results show that alcohol concentration, conversion and productivities presented no significant differences. Comparison of the TC composite biocatalysts with free cells showed that the composites gave higher ethanol concentration and therefore higher ethanol yield factors. The wines produced from mango and rice TC composites had a more distinguished aroma compared to the sal TC composite. This may be attributed to the presence of chemical extractive compounds present in these materials. All wines contained alcohol at concentrations more or less close to those of dry table wines. The free cell concentrations in the wines were low concluding that the fermentations were carried out primarily by the immobilized cells. Ethanol and wine productivities were more or less similar to those obtained in previous studies regarding wine fermentations using different biocatalysts (KANDYLIS et al., 2012). Fermentation at lower temperature might possibly affect the outcome of total and volatile acidities to be low compared with those at higher temperature.

The proposed biocatalysts are of food grade purity, cheap and easy to prepare, and they are attractive for bioprocess development based on immobilized cells. Such composite biocatalysts may be used for the co-immobilization of different microorganisms or enzymes (in separate layers of the biocatalysts), to efficiently conduct different types of fermentation in the same bioreactor, avoiding inhibition problems of chemical or biological nature, as discussed in the case of LAF.
Table 26. Kinetic parameters of the repeated batch fermentations of glucose and grape must at 15°C, using the Indian TC/alginate/PLA composite biocatalysts (TCC), plain TC biocatalysts, and free cells.

<table>
<thead>
<tr>
<th></th>
<th>Initial sugar (g/L)</th>
<th>Residual sugar (g/L)</th>
<th>Ethanol (% v/v)</th>
<th>Conversion (%)</th>
<th>Ethanol productivity (g/L/day)</th>
<th>Wine productivity (g/L/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Must</td>
<td>Glucose</td>
<td>Must</td>
<td>Glucose</td>
<td>Must</td>
<td>Glucose</td>
</tr>
<tr>
<td>Mango TCC</td>
<td>158.1</td>
<td>161.5</td>
<td>11.3</td>
<td>10.1</td>
<td>9.5</td>
<td>8.7</td>
</tr>
<tr>
<td>Rice TCC</td>
<td>161.5</td>
<td>161.5</td>
<td>10.4</td>
<td>10.5</td>
<td>8.5</td>
<td>8.1</td>
</tr>
<tr>
<td>Sal TCC</td>
<td>159.8</td>
<td>159.8</td>
<td>11.1</td>
<td>10.6</td>
<td>9.1</td>
<td>8.4</td>
</tr>
<tr>
<td>Mango TC</td>
<td>157.8</td>
<td>160.2</td>
<td>10.7</td>
<td>8.4</td>
<td>7.3</td>
<td>8.4</td>
</tr>
<tr>
<td>Rice TC</td>
<td>160.6</td>
<td>159.7</td>
<td>12.2</td>
<td>9.7</td>
<td>8.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Sal TC</td>
<td>160.2</td>
<td>159.9</td>
<td>8.6</td>
<td>7.2</td>
<td>7.8</td>
<td>8.1</td>
</tr>
<tr>
<td>Free cells</td>
<td>161.3</td>
<td>161.1</td>
<td>6.2</td>
<td>5.7</td>
<td>7.5</td>
<td>6.6</td>
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</table>
3.7.2.2 Formation of volatile by-products

According to previous studies (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014) natural carriers for cell immobilization such as delignified cellulosic materials (TCs), gluten pellets, barley grains, fruit pieces, etc., act as promoters of alcoholic fermentation even at very low temperatures (0-5°C), with much higher fermentation rates and productivities than free cells. The qualitative results of these studies have shown that the wines produced either with free or immobilized cells had improved aroma with similar profiles of volatile constituents at the various temperatures studied.

In this study quantification of the volatile compounds produced by the Indian origin TC biocatalysts was done and the results are presented in Table 27. The main volatile groups of compounds produced during fermentation were alcohols, esters and acids, all having a strong influence on wine aroma and alcoholic beverages in general, and subsequently, on product quality.

Table 27. Volatile compounds (mL/L) in the fermentation products of grape must and glucose media at 15°C using the Indian TC/alginate/PLA composite biocatalysts and free cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>KI</th>
<th>Grape Must</th>
<th></th>
<th></th>
<th>Glucose</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free cells</td>
<td>Mango</td>
<td>Rice</td>
<td>Sal</td>
<td>Mango</td>
<td>Rice</td>
</tr>
<tr>
<td><strong>Esters</strong></td>
<td></td>
<td></td>
<td></td>
<td>----------</td>
<td></td>
<td></td>
<td>----------</td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>518</td>
<td>-</td>
<td>-</td>
<td>0.21</td>
<td>-</td>
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<td>0.01</td>
</tr>
<tr>
<td>Vinyl acetate</td>
<td>562</td>
<td>-</td>
<td>-</td>
<td>0.15</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>Propyl acetate</td>
<td>694</td>
<td>-</td>
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<td>0.08</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>Ethyl propanoate</td>
<td>717</td>
<td>-</td>
<td>0.023</td>
<td>-</td>
<td>0.014</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>864</td>
<td>-</td>
<td>3.71</td>
<td>0.71</td>
<td>1.21</td>
<td>1.69</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethyl Butanoate</td>
<td>1040</td>
<td>-</td>
<td>0.18</td>
<td>0.07</td>
<td>0.26</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Ethyl Hexanoate</td>
<td>1218</td>
<td>-</td>
<td>0.134</td>
<td>0.03</td>
<td>0.07</td>
<td>0.90</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>1394</td>
<td>3.01</td>
<td>11.6</td>
<td>-</td>
<td>9.14</td>
<td>2.71</td>
<td>-</td>
</tr>
<tr>
<td>Methyl nonanoate</td>
<td>1552</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.03</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>1652</td>
<td>1.09</td>
<td>6.71</td>
<td>-</td>
<td>4.62</td>
<td>4.02</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl dodecanoate</td>
<td>1696</td>
<td>0.15</td>
<td>0.0315</td>
<td>-</td>
<td>0.015</td>
<td>0.021</td>
<td>-</td>
</tr>
<tr>
<td>Methyl benzoate</td>
<td>1777</td>
<td>0.51</td>
<td>1.25</td>
<td>0.02</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2-Phenylethylacetate</td>
<td>1847</td>
<td>2.59</td>
<td>4.26</td>
<td>0.1</td>
<td>2.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl nonadecanoate</td>
<td>2171</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.14</td>
<td>-</td>
</tr>
<tr>
<td>Isopropyl hexadecanoate</td>
<td>2216</td>
<td>-</td>
<td>0.0731</td>
<td>0.091</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total Esters</strong></td>
<td></td>
<td></td>
<td>7.35</td>
<td>27.97</td>
<td>1.46</td>
<td>18.30</td>
<td>9.46</td>
</tr>
</tbody>
</table>

| **Alcohols**            |     |            |          |          |         |          |          |          |
| 3-Penten-1-ol           | 840 | 0.27       | 0.011    | -        | -       | -        | -        | -        |
| Ethanol                 | 926 | -          | 5.21     | 0.87     | 3.25    | 2.91     | 0.76     | 1.37     |
| 1-Propanol              | 1011| -          | -        | 0.21     | -       | -        | -        | -        |
| 2-methyl-3-butanol      | 1184| -          | 1.42     | 0.7      | -       | 1.21     | 0.2      | -        |
| Isopentyl alcohol       | 1226| -          | -        | -        | -       | 0.91     | -        | -        |
| 2-Heptanol              | 1297| -          | 1.72     | 1.05     | 2.76    | -        | -        | -        |
| 1-Butanol               | 1320| 0.114      | 0.014    | 0.06     | -       | -        | -        | -        |

168 | P a g e
## 3. Results & Discussion

<table>
<thead>
<tr>
<th>Compound</th>
<th>KI</th>
<th>Grape Must</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free cells</td>
<td>Mango</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>1372</td>
<td>-</td>
<td>1.87</td>
</tr>
<tr>
<td>2-Undecanol</td>
<td>1418</td>
<td>-</td>
<td>3.87</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>1445</td>
<td>0.079</td>
<td>0.014</td>
</tr>
<tr>
<td>2-Nonanol</td>
<td>1475</td>
<td>-</td>
<td>3.16</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>1506</td>
<td>-</td>
<td>8.29</td>
</tr>
<tr>
<td>2,3-Butandiol</td>
<td>1569</td>
<td>1.34</td>
<td>0.11</td>
</tr>
<tr>
<td>2-tetradecanol</td>
<td>1681</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylethyl Alcohol</td>
<td>1889</td>
<td>0.62</td>
<td>4.61</td>
</tr>
<tr>
<td>3-(methylthio) 1-propanol</td>
<td>1730</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>Decanol</td>
<td>1783</td>
<td>0.10</td>
<td>0.21</td>
</tr>
<tr>
<td>Dodecanol</td>
<td>1998</td>
<td>0.04</td>
<td>-</td>
</tr>
</tbody>
</table>

Total Alcohols

|               | 2.80 | 26.63 | 9.31  | 13.51 | 14.50 | 3.34  | 6.79 |

### Organic acids

<table>
<thead>
<tr>
<th>Acid</th>
<th>KI</th>
<th>Grape Must</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free cells</td>
<td>Mango</td>
</tr>
<tr>
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<tr>
<td>Heptanoic acid</td>
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<td>-</td>
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<tr>
<td>Octanoic acid</td>
<td>2051</td>
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<td>6.89</td>
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</table>

Total Organic Acids

|               | 30.85 | 110.32 | 91.48 | 123.52 | 104.22 | 74.03 | 97.23 |

### Carbonyl compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>KI</th>
<th>Grape Must</th>
<th>Glucose</th>
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<tr>
<td></td>
<td></td>
<td>Free cells</td>
<td>Mango</td>
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<tr>
<td>Acetone</td>
<td>530</td>
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<td>2-Butanone</td>
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</tr>
<tr>
<td>2-Undecanone</td>
<td>1542</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Total Carbonyl compounds

|               | 0.39 | 0.82 | 2.53 | 0.65 | 0.14 | 0.42 | 1.77 |

Total compounds detected

|               | 41.39 | 116.75 | 169.91 | 157.86 | 128.32 | 79.22 | 117.3 |

Total no. of compounds

|               | 18 | 28 | 34 | 22 | 18 | 20 | 19 |

From the table above it can be concluded that all TC composite biocatalysts led to much higher concentrations of volatiles than free cells. The mango composite resulted in much higher concentrations of esters and alcohols than sal and rice. Likewise, grape must fermentation led to higher concentration of esters and alcohols in comparison with glucose synthetic medium. The sal composite was the least influential carrier regarding formation of esters and alcohols. In conclusion mango TC composite was the best carrier regarding the formation of volatile compounds that can positively affect the flavor of alcoholic beverages.
3. Results & Discussion

Chapter 8 - Conclusions

3.8.1 Conclusions

Renewable biomasses from agricultural wastes such as wood sawdust and rice husk are interesting alternatives of cellulose sources for several applications. These materials are cheap, abundant, and easily available around the world and are generally underutilized as burning fuel or animal feed or just disposed for ambient degradation. Delignification by alkaline treatment was used to extract the delignified porous cellulose (TC) from wood sawdust and husk of three plant species, i.e. *M. indica L.* (mango), *S. robusta* G. (sal), and *O. sativa L.* (rice).

The proximate analysis of leafs, bark and husk samples showed varying composition of the different plant species. TC extracted from sawdust and husk samples had higher cellulose content and lower hemicellulose and lignin contents than the original plant material. The SEM results illustrated that most of the TC extracted from the samples had tube diameters in the range of 10-15 µm. The results derived from the FTIR and NMR analyses confirmed that both lignin and most of the hemicellulose were removed during the chemical process. The crystallinity of extracted TC was higher than that of the non delignified sample.

The extracted TC revealed a two-step thermal decomposition behavior and the degradation temperature was between 300-400°C suggesting an enhanced thermal stability. In accordance with these results, the TC extracted from the wood sawdust of mango and sal and rice husk can find potential applications as fillers in paper and composites, water absorbents, or as raw materials for cellulose derivatives because of their higher aspect ratio. The present work may provide a new approach for high utilization of TCs and cellulosic microfibers for numerous bioprocess applications and can be further used for research on enzyme storage in order to increase activity for longer period and for biocatalysis during bioprocessing (KOUTINAS et al., 2012).

TCs have been previously used efficiently as yeast immobilization carriers for alcoholic fermentation processes (KOURKOUTAS et al., 2004; BEKATOROU et al., 2014; KOUTINAS et al., 2012), enhancing significantly the rate of alcoholic fermentation as compared to free cells. Therefore, their use as immobilization carriers was found to be more beneficial than the conventional method of the yeast immobilization (gel entrapment).

The concept to exploit the nano/micro tubing of TCs was developed from the idea to utilize this material as a porous, tubular matrix for cell entrapment as well as a microporous filter for liquid food clarification from microbial load. The above concept is being put into action considering the following aspects; firstly the valorization of renewable resources and secondly environmental safety by waste minimization. Therefore, wood sawdust has drawn the attention of researchers as it is a naturally occurring, renewable and biodegradable material (TROMBETTA et al., 2011).

The application of nano/micro-TC was effective in removing yeast and bacteria from drinking water and milk (skimmed milk mainly) by immobilization of the microbial cells in its pores and tubes. The proposed system presented adequate operational
3. Results & Discussion

stability for a long period at low temperature using the mango and sal TC. These materials showed better filtering efficiency and microbial load removal from drinking water and skimmed milk. Since rice husk is a more delicate, soft cellulosic material, it was degraded faster during the regeneration process (hot water washing) resulting in poor removal of microbial cells after only two regeneration process in drinking water. The increase of the filter lengths increased the microbiological load removal in all cases.

The cold pasteurization of drinking water and milk did not substantially alter the color, or sensory properties, except for milk were after the filtration the presence of lactic acid was detected in the filtered sample. This was due to the high numbers of \textit{L. bulgaricus} cells contained in the deliberately contaminated milk and retained on the filter causing lactic acid fermentation to occur. However, the proposed process is considered to be advantageous over the conventional thermal pasteurization eliminating the risk of food quality deterioration caused by heating. At the same time, undesirable microorganisms can be effectively removed with low energy consumption. The results encourage scale-up of the process, further improvements by development of cellulose-based biocomposites and more detailed research for application to other types of liquid foods.

Furthermore, use of \textit{L. bulgaricus} cells immobilized on mango, sal and rice husk TCs and their TC/Ca-alginate/PLA composites for lactic acid fermentation of lactose and cheese whey, led to increased rates and yields compared to free cells. No significant differences were observed among the different immobilized biocatalysts. The results are attractive for further research on bioprocess development based on cells immobilized on composite biocatalysts made from food grade purity and low cost materials. Such two or three-layer biocatalysts may be used for the co-immobilization of different microorganisms or enzymes to efficiently conduct different types of fermentations in the same bioreactor.

Finally, the study of alcoholic fermentation using the mango, sal and rice husk TCs as yeast immobilization carriers was evaluated. The results of repeated batch fermentations revealed that the TC biocatalysts exhibited differences in aroma, flavor and turbidity of the fermented liquids. Some of the volatile compounds detected derived from the Indian origin TCs giving a distinct character to the fermentation product. Additionally, the TC biocatalysts produced lower fused alcohol content and acidity. Comparatively rice husk TC was found to have much stronger aroma impact than the other two sawdusts. TC in general constitutes a cheap and ubiquitous support of food grade purity for yeast immobilization, performing fast fermentations making it cost effective and economic and can further produce a different quality of wines.

3.8.2 Scientific and technological consideration of results and perspectives

The results from cold pasteurizations clearly show that length of the filter affects the microbial load removal. Higher filter length improves the effective removal of microorganisms from the drinking water and skimmed milk. The filter source, i.e. TC from different plant species, also plays an important role in the operational stability
and mechanical strength during its regenerations. SEM images indicated the presence of tubular structures with cell entrapment within the tubes.

Cell entrapment within the cellulosic tubes was further utilized in successive batch fermentations to produce lactic acid from industrial wastes such as cheese whey. Alcoholic fermentation of grape must and glucose medium was also effective using the Indian TCs with entrapped cells. The presence of volatile compounds derived from the different TCs (i.e. sawdust and husk) also contributes towards end products with distinctive aroma.

The continuous cold pasteurization process results provided the elements to calculate that an industrial cold pasteurization process consisting of two filters of 5 m$^3$ can pasteurize a fair amount of liquid foods for example wine of 50,000 L in 5 days.

The TC filters can effectively remove yeasts and small sized bacteria, which exist in drinking water, and milk microflora. The ability of TCs to remove microbial cells with very big differences of cell dimensions can be attributed to the different size of pores and tubes in the TCs. These results can encourage scale-up of the process which has been already designed for the cold pasteurization of wine and beer. This detailed study of cold pasteurization will be a precursor investigation for other liquid foods such as beer, fruit juices, milk and liquids in industrial food processing that need pasteurization. The cold pasteurization process could possibly be a part of the microbial stabilization of industrially produced food or other materials for biomedical applications.

The transformation of the entire liquid food processing and dairy industry in the near future is targeted in micro/nanotechnology. Most of the potential applications are still under progressive investigation and evaluation. However, there are still few debates especially in concern to the accidental or deliberate use of nanoparticles in food, or food-contact materials, that consumers are mainly concerned about their potential negative effects on human health and also their regulatory stands. Several critical challenges, including discovering of beneficial compounds, establishing optimal intake levels, developing adequate food delivering matrices, product formulations and safety of the products still need to be addressed. An extensive review of science related to microbiological safety indicates that irradiation is a technology that can be an effective solution to the problem of microbial contamination. Under good manufacturing practices, irradiation can eliminate the harmful bacteria that can cause food poisoning and food spoilage. Irradiation is endorsed as a food safety process by independent health organizations and regulatory agencies around the world (LESTER & ERIC, 1996). But even this current technology took more than 5 decades of research and safety assessment for its acceptance in food and dairy processing. Similarly, micro and nanotechnology employing porous lignocellulosic biomasses also should also have to wait till all safety and biocompatibility issues are resolved. There is an urgent need for regulation of nanomaterials before their incorporations into liquid food and dairy processing area. Micro/nano materials must not cause any health risks for consumers or to the environment. More research studies are required to investigate the hazards of these materials and their origin specific sustainability.
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4. References


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Lactic acid fermentation by cells immobilised on various porous cellulosic materials and their alginate/poly-lactic acid composites

Mrinal Nishant Kumar, Angelika Ioanna Gialleli, Jean Bernard Masson, Panagiotis Kandylis, Argyro Bekatorou, Athanasios A. Koutinas, Maria Kanellaki

1. Introduction

For industries where competition is intense, innovation for improvement of production efficiency, food safety and food quality is vital. For that reason, research on bioprocess technology development has been extensive during the last decades. Specifically, the considerable scientific and industrial interest in applications of immobilised cell techniques for alcoholic and other food and fuel-related fermentation processes is due to the numerous advantages they offer compared to free (suspended) cell systems, such as the achievement of high cell densities in the bioreactors, higher productivities and shorter process times, protection against stress, feasibility of continuous processing, easy product recovery, reusability of the biocatalyst, etc. (Kourkoutas et al., 2004). Recently, nanotechnologies have also emerged as a potential aid for improvements in food production (Koutinas et al., 2012). The challenge for these technologies is to be usable at industrial scale with low production cost. Research needs to turn towards abundant and low cost materials with specific properties to create effective bioprocessing systems. Cellulose, which represents 30% of the lignocellulosic wastes from cotton, wood and paper industries, is one of these materials (Nikolov et al., 2000). Delignification of lignocellulosic substrates generates porous cellulosic materials (nano and micro-scale), in which microbial cells can be entrapped by growth into the formed cavities or by physical adsorption due to electrostatic and other weak forces (Kourkoutas et al., 2004). In numerous previous works, delignified cellulosic materials (or tubular cellulose, obbr, TC), have been used successfully as yeast immobilisation carriers leading to significant increase of fermentation...
rates as well as improvement of product quality (Koutinas et al., 2012; Kourkoutas et al., 2004; Mallocos et al., 2003). In these words, the TC biocatalysts exhibited all the advantages of immobilised cells. Moreover, they were found to decrease the activation energy \(E_a\) of alcoholic fermentation by ~28% compared to that of free cell systems, indicating that TC is an excellent material to promote the catalytic action of the immobilised cells (Koutinas et al., 2012). However, studies on the effect of TC lactic acid bacteria (LAB) biocatalysts on lactic acid fermentation of lactose containing waste streams, such as cheese whey, are limited (Koutinas et al., 2009).

Why is the major liquid product of the dairy industry and its disposal without any treatment a major source of environmental pollution. Its production in bulk quantities worldwide and its high organic load, make it a potential raw material for biotechnological conversion to various value-added products (Koutinas et al., 2008), such as lactic acid, which is widely used as natural preservative and acidity regulator in the food, pharmaceutical, leather and textile industries. Recently there is a sharp increase of lactic acid demand for the production of biodegradable polymers such as polyactic acid (PLA), which is hindered by the high prices of lactic acid. Therefore, reduction of lactic acid production cost by improvement of the productivities of lactic acid fermentation processes and utilisation of cheap substrates has become imperative (Abdel-Rahman et al., 2013). To improve the rate of whey fermentation for lactic acid production, the use of various TCs and TC/GA-alginate/PLA composites as LAB immobilisation carriers is proposed. The composite biocatalysts can be prepared in larger controllable sizes and the inclusion of the anaerobic LAB in the gel (alginate) matrix is expected to enhance their viability rates. The use of PLA is proposed in order to reduce the dimension of the micro-tubes in the TCs, to the nano-scale if possible, as it was previously shown in the case of TC/starch gel and TC/poly- hydroxybutyrate (PHB) composites, by the formation of starch gel or PHB layers in the internal of the TC tubes thus decreasing their volume. Using cells entrapped in such composite materials was found to increase the rate of biochemical reactions and give the possibility for different species immobilisation to conduct different processes in the same bioreactor (Koutinas et al., 2012; Servetas et al., 2013; Barouni et al., 2013). Therefore, the aim of the present study was to evaluate the possible promotional effect of TCs of various origins (Mango, Sal and Rice) and their Ca-alginate/PLA composites on lactic acid fermentation of lactose media and cheese whey.

2. Methods

2.1. Microorganisms and materials

The strain Lactobacillus delbrueckii subsp. bulgaricus DSMZ 20081 (DSMZ, Germany) was grown at 37 °C by repeated subculturing (static cultures) in 500 mL of medium containing 55 g/L MRS broth until enough cell mass was obtained. The cells were harvested by centrifugation at 5000 rpm for 8 min to obtain a cell mass of ~70% moisture. Cheesewhey (~50 g/Lactose/L) was supplied by Chelmos S.A. dairy industry (Santamari, Achaia, Greece). The synthetic media contained (g/L): lactose 20, yeast extract 5.0, KH2PO4 2.0, sodium acetate trihydrate 5.0, trinatrium citrate 2.0, MnSO4 0.05, and MgSO4 0.02. Sawdust from Mango wood (M. indica), Sal wood (S. robusta) and Rice husk were brought from a local timber producer in the Ranchi area, India. An amount of 300 g of each material were boiled for 3 h in 3 L of 15%w/v NaOH solution for lignin removal. The delignified materials (TCs) were thoroughly washed with water, drained and stored at 5 °C. Before use, they were dried overnight at 37 °C.

2.2. Cell immobilisation and lactic acid fermentation experiments

An amount of 5 g of each TC material (plain or composite) was added in a conical flask containing 300 mL of MRS broth and the whole was sterilised by autoclaving at 120 °C for 15 min. After cooling at 40 °C, 1 g of harvested L. bulgaricus culture was added in each flask and the mixtures were incubated at 37 °C for 48 h to allow cell immobilisation by natural adsorption and growth in the TC pores. For the preparation of the composites, the TC biocatalysts with immobilised L. bulgaricus and 0.2 g of polyactic acid (PLA) were mixed in 50 mL of a 25w/v Na-alginate solution. The mixture was then added drop-wise in 50 mL of a CaCl2 solution (15w/v) to obtain solidified composite beads. Lactic acid fermentation of 300 mL of cheese whey or lactose synthetic media was carried out at 37 °C using 5 g of the immobilised biocatalysts. Fermentation of whey was also carried out using 1 g of harvested culture (free cells) for comparison.

2.3. Analytical methods

Samples of the immobilised TC biocatalysts were washed with distilled water and dried overnight at 30 °C. The samples were then coated with gold in a Balzers SCD 004 sputter coater for 3 min and examined in a FEI model JSM-6300 scanning electron microscope. Structural characteristics of the TCs such as the Bruker, Emmitt and Teller (BET) specific surface area, Barrett, Joyner and Halenda (BJH) pore volume and average pore diameter, after degassing at 30 °C for 240–480 min under vacuum at 10 μm Hg, were measured at 196.15 °C using a Micromeritics TriStar 3000 porosimeter. Lactic acid was analysed on a Jasco LC-2000 Series HPLC system (Jasco Inc., Japan) equipped with a Bio-rad Aminex HPX-87H column (300 × 7.8 mm Id., 9 μm particle size), a CO-2060 PLUS column oven, a PU-2089 pump, an AS 2050 PLUS autosampler and a MD-2018 Photodiode array detector operated at 210 nm. Isocratic separation at 50 °C was performed with 0.008 N H2SO4 as mobile phase at a flow rate of 0.6 mL/min. The volatile constituents of the fermentation products were isolated by headspace solid phase micro-extraction (SPME) and were determined by GC/MS on a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer as described in Kandiyi et al. (2010). Semi-quantitative analysis was based on the peak areas of the identified compounds and the peak area of the internal standard (methyl octanoate, 10 μg/L). The significance of differences in the means of various groups was checked by One-Way Analysis of Variance (ANOVA) at 5% level of significance.

3. Results and discussion

The suitability of TCs of various origins (Indian Mango, Sal and Rice) and TC/GA-alginate/PLA composites as immobilisation carriers for L. bulgaricus cells for use in lactic acid fermentation of lactose media and whey was investigated. Initially, structural characteristics of the different TCs were analysed (BET specific surface area, cumulative pore volume and average pore diameter). The BET surface of the Rice and Mango TCs were similar (1.5 and 1.6 m2/g, respectively) and higher compared to the Sal TC as shown in Table 1. These values are similar to other porous materials that have been previously used for similar applications such as γ-alumina (1.40 m2/g) (Koutinas et al., 1991; Kourkoutas et al., 2004; Synigridis et al., 2013). However, the average cumulative pore volume of all TCs (0.008–0.037 cm3/g) was smaller compared to γ-alumina (0.45 cm3/g). Finally, the average pore diameter of Mango TC was higher, followed by Sal and Rice. Cell immobilisation on the TC materials and their Ga-alginate/PLA composites was observed by SEM and validated by the ability of the
biocatalysts to perform lactic acid fermentation of whey (50 g lactose/L) and synthetic lactose media (20 g lactose/L). The results are summarised in Table 2. The conversion of lactose was higher than 80% in all cases, but both free and immobilised cells performed better in the lactose media than in cheese whey, leading to higher conversion rates and higher lactic acid concentrations, yields (g lactic acid/g sugar utilised) and productivities (g lactic acid per L total working volume and per day). This may be due to lower substrate inhibition, since the lactose concentration in the synthetic media was lower, or more possibly to the complete medium composition compared to cheese whey which was not enriched by any nutrient addition. Lactic acid yields and productivities were significantly higher in the case of the immobilised cells compared to free cells (at the P < 0.05 level). Among the different immobilised systems used, the best results were obtained in the case of both plain and composite Mango TC biocatalysts. However, the differences observed between all the immobilised biocatalysts were not statistically significant (P < 0.05). The better performance of the immobilised L. bulgaricus cells may be attributed to the protective effects of the immobilisation matrix against stress, shearing forces and inhibitory effects of substrates, media components and fermentation products, as well as to altered physiology and metabolic activity in general due to immobilisation as discussed above.

The results of the semi-quantitative SPME-GC-MS analysis of the headspace volatile compounds of the products of lactose and whey fermentation by immobilised L. bulgaricus cells on the different TC/Ca-alginates/PLA composites are shown in Table 3. The analysis was performed to evaluate the effect of the immobilised biocatalysts on the formation of volatile by-products that may affect the quality of a fermented product destined for food

| Table 1 | Surface characteristics of Mango, Rice and Sal TCs. |
|---------|------------------|------------------|------------------|
|         | Mango | Rice | Sal |
| BET surface (m²/g) | 1.5 ± 0.5 | 1.6 ± 0.6 | 0.6 ± 0.3 |
| pore volume (cm³/g) | 0.006 ± 0.002 | 0.037 ± 0.015 | 0.002 ± 0.001 |
| BJH adsorption average pore diameter (Å) | 174 ± 1.7 | 104 ± 3.5 | 156 ± 4.4 |

| Table 2 | Lactic acid production during batch fermentations of cheese whey and lactose media at 37 °C using free and immobilised L. bulgaricus cells on Mango, Rice and Sal TCs and their composites (TCs/Ca-alginates)/PLA. |
|---------|------------------|------------------|------------------|------------------|------------------|------------------|
|         | Whey | Lactose | Residual sugar (g/L) | Conversion (%) | Lactic acid yield (g/L) | Lactic acid productivity (g/L) |
| Mango TC | 23.1 | 28.1 | 9.1 | 4.8 | 81.8 | 90.4 | 0.46 | 0.56 | 5.78 | 7.03 |
| Rice TC | 22.9 | 20.4 | 9.6 | 5.1 | 80.4 | 85.8 | 0.46 | 0.53 | 5.73 | 6.02 |
| Sal TC | 22.1 | 24.1 | 10.1 | 5.9 | 79.8 | 88.2 | 0.44 | 0.48 | 5.53 | 6.03 |
| Mango TC | 27.1 | 30.2 | 4.5 | 3.0 | 91.0 | 92.2 | 0.54 | 0.59 | 6.78 | 7.55 |
| Rice TC | 23.1 | 27.7 | 6.6 | 3.7 | 82.8 | 92.7 | 0.46 | 0.55 | 5.78 | 6.03 |
| Sal TC | 23.8 | 28.5 | 9.1 | 4.9 | 81.8 | 90.2 | 0.48 | 0.57 | 5.95 | 7.33 |
| Free cells | 19.6 | 20.6 | 10.4 | 8.4 | 79.2 | 83.2 | 0.39 | 0.41 | 4.90 | 5.15 |

| Table 3 | Volatile compounds (mL/L) identified in the fermentation products of cheese whey and lactose media at 37 °C using free and immobilised L. bulgaricus cells on Mango, Rice and Sal TC/Ca-alginates/PLA composites. |
|---------|------------------|------------------|------------------|------------------|------------------|
|         | Retention index | Whey | Lactose | | |
| Free cells | Mango | Rice | Sal | | Mango | Rice | Sal |
| Ethanol | 864 | - | - | - | 2.04 | 0.50 | 0.94 | - | - | - |
| Ethanol acetate | 1394 | - | - | - | 0.42 | - | - | - | - | - |
| Methyl nonanoate | 1552 | - | - | - | - | - | - | - | - | - |
| Methyl benzoate | 1777 | 0.34 | - | - | 0.19 | - | - | - | - | - |
| Methyl nonadecanoate | 1771 | - | - | - | - | - | - | - | - | - |
| 2-Phenylethyl acetate | 1784 | - | - | - | 0.39 | - | - | - | - | - |
| Butyl octanoate | 1838 | - | - | - | 0.96 | - | - | - | - | - |
| Ethanol | 926 | - | - | - | 3.10 | 0.99 | 2.57 | 0.94 | - | - |
| Isobutyl alcohol | 1226 | - | - | - | - | 0.21 | - | - | - | - |
| 3-Methyl-2-butanol | 1317 | - | - | - | - | - | - | - | - | - |
| 1-Butanol | 1445 | - | - | - | 0.84 | 0.36 | - | - | - | - |
| 2-Nonanol | 1475 | - | - | - | 1.48 | 0.72 | 3.22 | 4.01 | 5.75 | - |
| 1-Heptanol | 1488 | - | - | - | - | 0.27 | 0.81 | - | - | - |
| 2-Heptanol | 1497 | - | - | - | 0.73 | 1.19 | 1.40 | - | - | - |
| 1-Octanol | 1506 | - | - | - | 4.47 | 1.33 | 1.82 | - | - | - |
| 2-Hexadecanol | 1678 | - | - | - | 5.49 | 3.43 | 2.81 | 1.57 | 2.42 | - |
| 2-Tetradecanol | 1684 | - | - | - | - | 2.61 | - | - | - | - |
| Phenylpropyl alcohol | 1889 | 0.05 | - | - | 3.43 | - | - | - | - | - |
| Acrinal alcohol | 1424 | - | - | - | - | - | - | - | - | - |
| Hexanol | 1818 | - | - | - | 52.43 | 24.34 | 22.57 | 8.51 | 6.22 | 1.83 |
| Octanol | 2051 | - | - | - | 3.73 | 96.75 | 93.11 | 8.47 | 7.51 | 17.98 |
| Decanol | 2247 | 45.50 | - | - | 42.74 | 36.84 | 30.47 | - | - | - |
| Hexanal | 1067 | - | - | - | - | 0.25 | - | - | - | - |
| 2-Heptanone | 1172 | - | - | - | 0.25 | 0.41 | - | - | - | - |
| 2-Decanone | 1344 | - | - | - | - | - | - | - | - | - |
| Benzoylefale | 1477 | 0.39 | - | - | 1.34 | 0.72 | - | - | - | - |
| 2-Isononanoate | 1542 | - | - | - | 0.44 | 0.17 | - | - | - | - |
| Total | 47.08 | 116.75 | 169.91 | 157.86 | 33.48 | 28.17 | 61.63 | - | - | - |
production. Alcohols, esters, carbonyl compounds and organic acids that can potentially influence the flavour of a fermented food product were detected. In the case of cheese whey, the fermentation products of the immobilized cells presented a more complex profile and higher amounts of volatile by-products compared to free cells as previously shown (Mallouchou et al., 2003; Servetas et al., 2013; Plessas et al., 2007). Moreover, higher amounts of volatile compounds were found in the fermented whey samples compared to the synthetic lactose media, a number of which may be derived from the substrate (Katechaki et al., 2010) and the rest are fermentation by-products. The major volatiles produced were organic acids and alcohols. Esters were also produced, which can affect a fermented product flavour as they usually have low odour threshold values and pleasant flowery and fruity aromas (Mallouchou et al., 2003). The other groups of compounds generally contribute to the complexity of the aroma, except specific compounds such as phenylethanol, benzaldehyde, etc., with characteristic odours and low threshold values.

4. Conclusion

Using L. bulgaricus cells immobilised on Mango, Sal and Rice TCs and TC/Ca-alginate/PLA composites, the fermentation rates and yields of lactic acid fermentation of lactose media and cheese whey were increased, compared to free cells. No significant differences were observed among the different immobilised biocatalysts. The results are attractive for further research on bioprocess development based on cells immobilised on composite biocatalysts made from food grade purity and low cost materials. Such two or three-layer biocatalysts may be used for the co-immobilisation of different microorganisms or enzymes to efficiently conduct different types of fermentations in the same bioreactor.

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References


Nano and micro-tubing performance in wheat flour gluten macromolecule for bioprocessing

Gialleli Angelika-Ioanna, Kumar Nishant Mrinal, Bekatorou Argyro, Kanellaki Maria, Koutinas Athanasios

Food Biotechnology Group, Department of Chemistry, University of Patras, 26500, Patras, Greece, e-mail: alina86gr@yahoo.gr

ABSTRACT

The field of nanobiotechnology and its application in food sector has drawn the attention of scientific research, targeting on the improvement of quality and industrial processes. In the present study, a natural biopolymer, gluten is treated in the form of spheres in order to obtain nano and micro tubes for bioprocessing. The tubing is achieved after treatment with lignin and the formation of pores was assessed after lignin removal. For this purpose, gluten samples were treated with aqueous solution of lignin sodium salt, isolated from sawdust delignification process. Then gluten samples were treated with H₂SO₄ 4% and they were thermally dried in a chamber at 105°C for five hours. The effect of temperature and treatment duration time on tube formation was examined. To improve tube formation, gluten samples were treated with alcoholic solution 75% of lignin 2 g/L at 25°C for various periods of time and then washed with NaOH 1%. The size and shape of pores were studied with BET porosimetry and Scanning Electron Microscopy (SEM). The samples analysis showed satisfactory tube formation at all temperatures and in general bet surface increased along with the treatment duration time.
Micro and Nano-tubing Processes for Biopolymers to be Employed for Food Cold Pasteurization and Biocatalysis

Athanasios A. Koutinas*, Angelika-Ioanna Gialleli, Mrinal Nishant Kumar, Argyro Bekatorou, and Maria Kanellaki

Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

ABSTRACT

In the introductory part of this investigation a short literature review of research breakthroughs in the field of Micro and Nano-biotechnology and mainly those of cellulose Nano-biotechnology will be reported. Furthermore, the attempt to improve micro and nano-tubing of lignocellulosics and to obtain micro and nano-tubes in microbial biopolymers such as PHB and PLA will be discussed. In a recent study concerning formation of micro and nano-tubes in the mass of cellulose, it was shown that this material is suitable for bioprocess development such as cold pasteurization of liquid foods (as filter), promotion of the rate of alcoholic fermentation, and feasibility of extremely low temperature fermentations. However, the process for tubing of cellulose (by lignin removal) resulted to a BET surface of about 1 m$^2$/g. In order to develop new materials for use in bioprocessing that have increased micro and nano-tubing levels, various microbial polymers were also evaluated. Tubing in these biopolymers was done by development of wet and dry processes which are discussed in the present investigation along with a study of cellulose structural characteristics based on its origin. The wet process involved impregnation of biopolymers in a lignin sodium salt solution, formation of solid lignin by treatment with sulfuric acid and then removal of lignin with 1% NaOH solution. The dry process involved treatment of cellulose with N$_2$ under high temperature and pressure. The BET surface, pore volume and pore diameter were measured and the formation of tubes was studied by Scanning Electron Microscopy, Properties of tubular cellulose, PLA and PHB were examined by X-ray diffractometry and TGA analysis. Cellulose samples of Indian and Greek origin showed a differentiation of BET surface in the level of 400%. The carbonization of cellulose at high temperature and N$_2$ flow, resulted to an increase of BET surface higher than 30 fold and a substantial increase of nano and micro tubes. Likewise, the effect of the increase of the level of micro and nano tubing of these biopolymers on biocatalysis (alcoholic and lactic acid fermentations) and cold pasteurization of liquid foods (drinkable water, wine and beer) is discussed.

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Utilization of kefir SCP produced using agroindustrial wastes for yeast extract production

Theodoros Aggelopoulos\textsuperscript{a}, Argyro Bekatorou\textsuperscript{a}, Athanasios A. Koutinas\textsuperscript{a,*}, Mrinal Nishant Kumar\textsuperscript{a}, and Ashok Pandey\textsuperscript{b}

\textsuperscript{a}Food Biotechnology Group, Department of Chemistry University of Patras, Greece\textsuperscript{b}

\textsuperscript{b}Biotechnology Division

National Institute for Interdisciplinary Science and Technology, 695 019, Trivandrum, India

ABSTRACT

Agroindustrial wastes have been used in the present study for single cell protein (SCP) production in submerged (SmF) and solid state fermentation (SSF) using mixtures of whey, molasses, orange pulp and potato pulp for SmF and mixture of Brewer’s spent grains and malt spent rootlets, among the other wastes, for SSF. Sterilized substrates were inoculated with 1 g kefir biomass and let for fermentation for 4 days with air supply in SmF and without agitation in SSF. Yeast extract is the common name for various forms of processed yeast products made by extracting the cell contents (removing the cell walls). They are used as food additives or flavourings, or as nutrients for microorganisms’ culture media. The extraction of the cell contents was carried out by autolysis, a degradation process that produces soluble products of peptides, amino acids, nucleotides and amino acid derivatives and was performed as described by Tanguler & Erten, 2008. SCP was suspended in distilled water and pH level was adjusted to 6.0. Autolysis was carried out at controlled temperature of 50°C in a water bath for 24 h. The process was terminated by heating at 80°C for 30 min and then cooled down. The suspension was centrifuged to remove the cell debris. The supernatant was concentrated by using water bath at 80°C and the concentrate was then freeze dried to produce yeast extract powder.

Yeast extract powder that was received was analyzed for nitrogen and metal content (Ca, Mg, Fe and Cu) and then used as nutrient in glucose synthetic medium for S. cerevisiae growth, in three different concentrated (0.3, 0.4 and 0.5\%w/v). Commercial yeast extract was used in the same concentrations for comparison. The final biomass was determined after centrifugation and by optical density after the construction of a standard curve, measuring the absorbance of specific amount of S. cerevisiae biomass with spectrometer. Biomass productivity value was then determined and it was detected that the autolysed SCP of kefir produced by SmF gave higher value of biomass productivity than that of commercial yeast extract powder and the optimum concentration was 0.5\%w/v.

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Nano and the microtubing structures of the polylactic acid for the cold pasteurization of milk and drinkable water

Shramona Roy\textsuperscript{a,*}, Mrinal Nishant Kumar\textsuperscript{b}, Argyro Bekatorou\textsuperscript{b}, Angelika-Ioanna Gialleli\textsuperscript{b}, Athanasios A. Koutinas\textsuperscript{b}, Maria Kanellaki\textsuperscript{b} and Poonam Singh Nigam\textsuperscript{a}

\textsuperscript{a}School of Biomedical Science, University of Ulster, Northern Ireland, (U.K)
\textsuperscript{b}Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

ABSTRACT

The presence of renewable resource has developed and excellent idea to utilize the use of cheap raw material available ubiquitously in the nature for the commercial benefits. The exigency for the scientific amelioration and novel technologies in the area of the food biotechnology and fermentation technologies through nanotechnology has initiated a scope for research in the process of cold pasteurization. Polylactic acid (PLA), which is one of the most promising biodegradable polymers, is being used for this purpose through the creation of pores in its mass. The current process involves the treatment of 300 g of wood sawdust with 3L NaOH 1\% to be heated for 3 hours continuously. This delignification process is carried out in order to remove the lignin from the cellulose matrix of a wood sawdust sample. This proposes a formation of tubular structures of wood cellulose, which is further added to prepare a PLA-cellulose composite in a form of nano and micro structures that has been used for the cold pasteurization of milk and drinkable water. The surface structure of PLA-cellulose composite was examined using the methods of Porosimetry, X-Ray diffractometry (XRD), and Scanning electron microscopy (S.E.M). The liquid food to be pasteurized is passed through the column packed with PLA-cellulose composites under refrigerated conditions to achieve the principles of cold pasteurization. The microbial load in the liquid sample is being analyzed for the inflow and the outflow to evaluate the filtration strength of the continuous bio-reactor in regular time intervals. The current process was proved to be an effective treatment compared to conventional pasteurization process, as no external energy such as heat is provided to destroy the microbial load and degrade the nutritional properties of the liquid foods.
Biopolymers reinforced with Micro and Nano-tubular Cellulose of Indian Origin
For Use in Liquid Food Cold Pasteurization Processes
Kumar Mrinal Nishant*, Bekatorou Argyro, Gialleli Angelika-Ioanna, Koutinas A. Athanasios, Kanellaki Maria

Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

ABSTRACT

The need for innovative and sustainable technologies in the area of food bioprocessing and fermentation technology has brought upon a great interest on the use of cheap renewable resources in the form of raw materials available in their natural existence. Wood sawdust and rice husk are such materials that can be used as novel biodegradable micro and nanotubular cellulosics after a delignification process, alone or as composites with natural microbial biopolymers such as Polylactic acid (PLA) / Polyhydroxy butyrate (PHB). Micro and nano-structural materials consisting of tubular cellulose from Mangifera indica (mango tree), Shorea robusta (shal tree) wood sawdust, and rice husk of Indian origin were prepared and characterized in this study. Lignin was removed from the cellulose matrix by alkaline treatment to leave behind a tubular structure that can be used as micro/nanofilter for “cold pasteurization” of liquid foods. Lignin analysis was done by the Kalson method to determine the lignin content present in Indian & Greek wood sawdust before and after the delignification treatment. The micro/nanotubular structure of the prepared cellulosic materials was measured by BET surface analysis applied after nitrogen flow for various time intervals at a high temperature. Subsequently, the microbial biopolymers PHB and PLA were mixed with sodium alginate and solidified with Ca\(^{+2}\) to form PLA/Alginate and PHB/alginate beads that where further characterized by porosimetry, SEM and X-Ray diffractometry (XRD). In a similar manner, composites of tubular cellulose with PLA or PHB were prepared and a 2-L bioreactor packed with these materials was used for continuous cold pasteurization of drinkable water and wine. The microbial loads where analyzed before and after the process to estimate the filtration strength of liquid contaminated food.

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Promotional Effects of Tubular Cellulose on Lactic Acid Fermentation

Kumar Nishant Mrinal *, Angelika-Ioanna Gialleli*, Jean Bernard Masson2, Bekatorou Argyro 1, Koutinas A. Athanasios1, Kanellaki Maria 1

1Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece
2Polytech Clermont-Ferrand, Blaise Pascal University, France

ABSTRACT

Lignocellulosic biomass is a cheap and available material well known for its numerous applications in food bioprocessing and fermentation technology. In this study, tubular cellulose (TC) samples, produced by delignification of Magnifera indica (mango wood), Shorea robusta (shall wood) and rice husk of Indian origin, were evaluated as novel biodegradable micro/nanotubular cellulosic materials. Lignin was removed from the cellulose matrix by alkaline treatment in order to create a micro and nano tubular structure suitable for cell entrapment and to affect bioconversion rates during batch and continuous fermentation of cheese whey and synthetic media. Specifically, Lactobacillus bulgaricus was used for lactic acid fermentation of both cheese whey and synthetic lactose media in the presence of the different TC materials of Indian origin. The BET surface, pore volume, and pore diameter of the materials was also analyzed to compare their effect on the fermentation. TC samples with entrapped L. bulgaricus cells were examined by SEM after the end of the 5th fermentation batch of both cheese whey and synthetic media. Tubular cellulose supports with L. bulgaricus promoted lactic acid fermentation of whey and synthetic medium containing glucose. The Composite PLA/Alginate/TC biocatalyst promoted further lactic acid fermentation faster and in repeated batches than compared with the free cells. Finally, SPME GC/MS analysis of volatile compounds was performed in the products of whey and synthetic media fermentations with the composite PLA/Alginate/TC biocatalyst and with free cells for comparison.

Acknowledgements

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Cold pasteurization of drinkable water using micro and nano-tubular cellulose of indian origin

Mrinal N. Kumar 1, Argyro Bekatorou 1, Katerina Pissaridi 1, Athanasios A. Koutinas 1, and Maria. Kanellaki 1

1Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

ABSTRACT

The need for innovative and sustainable technologies in the area of food bioprocessing and fermentation technology has brought upon a great interest on the use of cheap renewable resources in the form of raw materials available in their natural existence. Wood sawdust and rice husk as an ubiquitous lignocellulosic biomasses are such materials that can be used as a novel biodegradable micro and nanotubular cellulosic's after a delignification process to design a natural microbial biofilters for liquid food cold pasteurization. Micro and nano-structural material consisting of tubular cellulose (TC) from Magnifera indica (mango tree), Shorea robusta (sal tree) wood sawdust and oriza sativa (rice husk) of Indian origin where used. Specific surface area and lignin content in TC were determined before and after the delignification treatment. The present work involves the use of these cellulosic materials with difference in microbial load concentration (0.2, 0.5 g/L) and filter length (35, 45cm) during the cold pasteurization of drinkable water at 4°C with flow rate 2L/day using a one-way peristaltic pump. Regeneration of the filters was done using hot water. The microorganisms used for contaminating drinkable water were Lactobacillus Bulgaricus and Saccharomyces cerevisiae AXAZ-1. The nano/micro TC filters of different origin where placed in a packed-bed bioreactor using a nylon thin fabric with stainless steel covers. Both inflow and outflow were analyzed using the standard plate count technique and spectrophotometry at 600 and 700nm. The efficiency of the mango and sal TC was higher than the rice husk TC in all cases. The microbial removal load ranged from 80-100 % for S.cerevisiae and 70-90 % for L.bulgaricus.

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Preparation and characterization of porous biopolymer materials and their use for food bioprocess development

M. N. Kumar*, A. Bekatorou, A. A. Koutinas, M. Kanellaki

Food Biotechnology Group, Department of Chemistry, University of Patras, Patras 26500, Greece

ABSTRACT

Porous materials based on delignified tubular cellulose (abbr. TC) from Mangifera indica (mango tree), Shorea robusta (sal tree) sawdust and Oryza sativa (rice) husk of Indian origin, and their composites with microbial polymers (Ca-alginate and polylactic acid) were prepared and used in various food bioprocess. Carbonization of the TC’s under nitrogen flow was also done at various temperature/time protocols to increase their specific surface area. All materials were analyzed by TGA/DTA, SEM and BET, and were evaluated as Lactobacillus bulgaricus immobilization carriers for lactic acid fermentation. Both type of biocatalysts (cells immobilized on plain or composite materials) led to improvements in lactose and cheese whey fermentations compared to free cells, with lower fermentation rates, higher lactic acid yields and higher amounts of flavor-active volatile by-products. The TC materials were also evaluated as natural biofilters for “cold pasteurization” (not involving heat treatment) of intentionally contaminated drinkable water and semi-skimmed milk in a packed-bed bioreactor at 4 °C operating continuously. The effects of initial microbial load (L. bulgaricus and Saccharomyces cerevisiae; 0.2-0.5 g/L) and filter length (35-45 cm) on cell removal were evaluated. The filters were regenerated by washing with hot water. Microbial load removal (MLR) was assessed by colony forming units and OD (600, 700 nm) measurements. The efficiency of the mango and sal TCs was higher than the rice husk TC in all cases. The microbial load removal was 80-100% for S. cerevisiae and 70-90% for L. bulgaricus. The composite biocatalysts can be used for co-immobilization of different species or enzymes, to simultaneously conduct different types of fermentations without inhibition (biological competition) problems.

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