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# Extraction of Chitin from Shrimp Shell Wastes by Using Bacillus Licheniformis and Lactobacillus Plantarum

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*Abstract*- **The present study deals with biological extraction of chitin from shrimp shell waste were done, as an alternative method for chemical extraction to retain the quality of chitin. The shrimp shell waste was deproteinised by using immobilised Bacillus licheniformis (SSCL10) and Lactobacillus plantarum (MTCC 1407) was used for demineralisation of shrimp shell waste for chitin extraction. Maximum level of extracellular protease (16 U/ml) was produced on the 4th day of fermentation. The protein content of shrimp shell was greatly reduced to 3% from 25% on the 7th day. Maximum deproteinisation of shrimp shell waste of 90% was by B. licheniformis and maximum demineralisation of 96% was observed on the 7th day by using Lactobacillus plantarum (MTCC 1407).The chitin extraction from shrimp shell waste after deproteinisation and demineralisation showed 30% efficiency. The extracted chitin was confirmed by comparing it with the FT-IR spectrum with standard chitin. The Moisture content and ash content of obtained chitin were also characterized. The present study revealed that a better economic usage of the shrimp waste which minimizes the land pollution and obtained profitable chitin products.**

Key words: Deproteinisation, demineralisation, chitin, shrimp waste etc..

## INTRODUCTION

Shrimp industry in India has grown to a greater extent in the last few years due to excessive global demand. Asian countries accounts for 80% of the shrimp production, with India being the pioneer in the field (Kandra et al., 2012). Wang et al. (2007) reported that the carapace, tail and leg portions of crustacean are not being consumed and are removed during food processing which accounts up to approximately 50% of the harvested amount. This waste is a major source of pollution in coastal areas (Arvanitoyannis and Kassaveti, 2008). Van Ornum (1992) reported that utilization of shrimp waste for recovery of value added products like chitin, chitosan etc. would minimize the pollution problem and at the same time maximize the profits of the processors. Few attempts have been made to utilize shrimp waste as a source of protein, pigments (e.g. astaxanthin), flavour compounds (Handayani et al., 2008) and chitin (Redde et al., 2008).

Commercially, shrimp shell wastes are managed by treating them with strong acid and alkali to remove minerals and proteins respectively (Setoguchi et al., 2012). These chemical methods have brought more environmental hazards. Moreover, Wang et al. (2006) reported that chemical reactions are much difficult to control and require desalting and other processes. To overcome these problems caused by chemical treatments, alternative methods like use of microorganisms (Jo et al., 2008; Muzzarelli et al., 2012) and proteolytic enzymes (Valdez Pena et al., 2010) have been introduced. The optimization and standardization of the extraction process is needed to produce high yield with minimum cost (Pal et al., 2014).

The efficiency of fermentation using microorganisms depends on the quantity of inoculums, the glucose concentration, the initial pH and pH during culture and fermentation time (Jung et al., 2005). The application of proteolytic microorganisms for deproteinisation and into useful bioactive substances of shrimp waste is the current research trend in bioremediation/environmental biotechnology. It is a simple and environmental friendly alternative methods employed in the chitin extraction process. In this paper, the performance and efficiency of Bacillus licheniformis (SSCL10) isolated from the shrimp contaminated soil from Thoothukudi area for deproteinisation and demineralisation of shrimp shell waste by Lactobacillus plantarum (MTCC1407) for chitin extraction was studied.

## MATERIALS AND METHODS

**1. Microorganisms**

Bacillus licheniformis strain (SSCL10) isolated from shrimp waste contaminated soil from Thoothukudi area from my previous work (Abirami et al.,2016 ) and it was maintained in the laboratory on Nutrient agar slant and stored at room temperature 28oC ± 2oC and routinely grown in Luria Bertani (LB) medium. It was further checked for purity and screened for the protease activity on skim milk agar medium. The isolate was used for deproteinisation of shrimp shell wastes. The bacteria Lactobacillus plantarum (MTCC 1407) was collected from Microbiology department, V.H.N.S.N. College, Virudhunagar, Virudhunagar district, Tamil Nadu, India. It was routinely grown in MRS medium and checked for acid production using glucose for chitin extraction. The isolate was used for demineralization of this study.

**2. Collection of shrimp shell waste**

 

Shrimp shell waste was collected from a seafood processing unit in Thoothukudi. The exoskeleton materials were peeled off from shrimp waste carefully with minimum tissue contamination and mixed with 70% saline solution for 3 hours to remove the unbound tissues and contaminating microorganisms. The saline treated shell waste was repeatedly washed with sterile distilled water and filtered using muslin cloth. The washed shell waste was dried under vacuum and powdered using blender.

**3. Biological method of chitin extraction**

The biological method of chitin extraction from shrimp shell waste was done according to the procedure of Pal et al. (2014).

**3.1. Immobilization of bacterial cells for Deproteinisation**

2% sodium alginate beads with entrapped Bacillus licheniformis (SSCL10) were prepared by the method of Bhagat et al., (2014). 2g of sodium alginate was dissolved in 100 ml of boiling water and autoclaved at 121oC for 15 minutes. Bacillus licheniformis (SSCL10) cells were harvested during the mid logarithmic growth phase by centrifugation (10,000 rpm, 10 min) and resuspended in 2% sterile alginate solution. The alginate/cell mixture was extruded drop by drop into a cold, stirring, sterile 0.2 M CaCl2 solution using a sterile 5 ml pipette in a Laminar Air Flow Chamber. Gel beads of approximately 5 mm diameter were obtained. The beads were hardened by resuspending into a fresh CaCl2 solution for 30 minutes at room temperature with gentle agitation. Finally, these beads were washed with sterile distilled water to remove excess calcium ions and unentrapped cells.

**3.2. Deproteinisation of shrimp shell powder (SSP) by immobilized bacteria**

The fermentation medium for deproteinisation of shrimp shell waste was prepared with the following composition: Shrimp shell powder-8.0 (g/L), Yeast extract -0.5 (g/L), NH4)2SO4-1.0 (g/L),MgSO4.7 H2O-0.3 (g/L),KH2PO4-1.36 (g/L) and pH 7.0

Thirty immobilized bacterial cell beads of uniform size were transferred to 300 ml of fermentation medium and was gently agitated at 180 rpm in a rotatory shaker at 30oC. Samples from fermentation medium for protein estimation were obtained at an interval of 24 h by collecting an aliquot of medium and centrifuging it at 8000 rpm for 20 minutes. The supernatant was filtered through a 0.2 µm cellulose microfilter and the filtrate was used for protease assay.

**3.3. Protease assay**

The activity of protease was estimated according to the procedure Ferrero (1996) and modified by Cheong et al. (2013). 150 µl of the filtrate was added to the reaction mixture with 750 µl of substrate casein at a concentration of 10 mg/ml in 200 mM of sodium phosphate buffer, pH 7.4 and 5 mM phenyl methyl sulphonyl fluoride (PMSF) and incubated in a water bath at 37oC for 30 minutes. Enzyme reaction was terminated by adding 150 µl of 10% trichloroacetic acid (TCA) in an ice bath for 15 minutes. The mixture was centrifuged at 8000 rpm for 5 min to precipitate undegraded protein. The supernatant (750 µl) was added with 3.75 µl of 0.5 M sodium carbonate and 750 µl Folin-Ciocalteau reagent (three fold diluted with distilled water) and incubated in dark for 2 h. The colour developed was measured at 660 nm with tyrosine as the reference compound. One unit of protease activity was expressed as the amount of enzyme required to release 1 µmol of tyrosine.

3.4. Estimation of protein content from deproteinized sample

The centrifuged pellet from deproteinized medium was dried and analyzed for protein content using Lowry's method to find out the degree of deproteinisation. The pellet was treated with 1 N NaOH (1:10 w/v) for 2 h at 65oC. The digested pellet was centrifuged at 10,000 rpm for 10 minutes to remove the non protein content and the supernatant was collected for protein estimation. Concentration of the protein was calculated by Lowry's method. Bovine serum albumin was used as standard. The colour developed using Folin-Ciocalteu reagent was measured at 670 nm in a spectrophotometer (Elico, India) and the protein value was expressed as %.

**3.4. Preparation of inoculum for Demineralisation**

Lactobacillus plantarum (MTCC 1407), a lactic acid producing bacterium was used for demineralisation of shrimp shell powder. L. plantarum (MTCC 1407) was maintained in MRS media. 2 ml of starter culture was transferred to 100 ml of sterile MRS broth and incubated at 30oC for 24 h and were used for demineralisation studies.

**3.4.1. Demineralisation of shrimp shell waste by Lactic acid bacteria**

Microbial extraction of chitin was obtained by slightly modified method of Synowiecki (1997).

**3.4.2. Optimization of media of demineralisation of shrimp shell waste by Lactic acid bacteria**

The conditions for chitin extraction from deproteinized shrimp shell powder was optimized with the effect of nitrogen variables (factors) such as peptone, yeast extract and Fe(NO3)3. 1% of each factor was added to (minimal medium) fermentation medium separately and their response on the chitin extraction was determined.

**3.4.3. Effect of glucose concentration on the demineralisation of shrimp shell waste**

In the demineralisation medium, 3g of deproteinated shrimp shell powder was mixed along with glucose concentrations of 5%, 10% ,15% and 20% and inoculated with 10% inoculum of L. plantarum (MTCC 1407) (v/w). After seven days of fermentation at 30oC, their effect on chitin extraction and also on pH was determined.

**3.4.4. Demineralisation of deproteinized shrimp shell powder**

The deproteinized shrimp shell powder (5 gm) was taken with 100 ml of minimal medium (containing 0.1% KH2PO4; 0.05% MgSO4.7H2O; 50mM sodium phosphate buffer) and 1% Fe (NO3)3. The pH was adjusted to 7. The 15% of glucose was added into the medium as a carbon source for acid production and sterilized at 1210 C for 15 minutes and cooled. Lactobacillus plantarum (MTCC 1407) of (OD600=0.8-1) culture was used as an inoculum. The inoculated medium was

 

mixed in a rotatary shaker at 50 rpm for 7 days at room temperature for fermentation. Glucose concentration and lactic acid content of the incubation mixture were analysed for every 24h. After 7 days of fermentation, sediments were removed, washed and the resultant solid fraction was dried in hot air oven for the estimation of chitin. The fermented shrimp waste was filtered to remove liquor fraction. The remaining solid chitin fraction was washed out, oven dried and used for further analysis.

$$DM = [AO \times O\text{-}AR \times R] \times 100/AO \times O$$

The demineralisation efficiency was calculated using the equation, whereas the O and R are the masses before and after fermentation respectively (g), the AO and AR are the ash contents of original and residue sample respectively (g/g).

### 3.4.5. Estimation of Lactic acid

The amount of lactic acid in medium was determined by transferring 25 ml of culture broth of LAB (Lactic Acid Bacteria) isolates into 100 ml flask. One ml of phenolphthalein indicator (0.5% in 5% alcohol) was added into the flask. This was titrated with 0.25 M NaOH for the appearance of pink colour. The titratable acidity was calculated as lactic acid % W/V (Sheeladevi and Ramanathan, 2011). Each millilitre of 1N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acidity was then calculated.

### 3.5. Estimation of Chitin

The chitin present in the deproteinated shrimp shell waste during demineralisation was quantified according to the method suggested by Babu et al. (2012). 10 ml of fermentation medium was taken for every 24h and centrifuged to remove the medium. To the pellet, 100% acetone was added and heated in a water bath for 1 hour in a test tube (with its mouth covered with aluminium foil) to its boiling point. After cooling, ethanol was added and shaken till the dissolution of precipitate. 0.25 ml of Ehrlich reagent was added to the above mixture and the colour developed was measured at 530 nm in a spectrophotometer. The amount of chitin in the sample was calculated from the standard graph drawn using commercial chitin.

### 3.6. Moisture content

Crude chitin sample (2g) was placed in a pre-weighted aluminum dish. The dish and contents were then placed in an oven at 105oC for 24h. The aluminium dish along with the dried sample was first placed in a desicator to cool down and then weighed. The moisture content was determined as follows (Mahmoud et al., 2007).

$$MC = \frac{Wws - Wds \times 100}{Wws}$$

Wherein: MC : the moisture content (%), Wws: the weight of the wet sample (g), Wds: the weight of the dry sample (g)

### 3.7. Analysis of ash content

To determine the ash value of residue, 2.0 g of chitin sample was placed into a previously ignited, cooled and tarred crucible. The samples were kept in a muffle furnace preheated to 650oC for 4h. The crucibles were allowed to cool in the furnace to less than 200ºC and then placed into desiccators with a vented top.

Calculation

$$\% \text{ Ash} = \frac{\text{(Weight of residue, g)}}{\text{(Sample weight, g)}} \times 100$$

Moisture content and ash content was done at Chemistry Department, V.H.N.S.N. College, Virudhunagar, Virudhunagar district, Tamil Nadu, India.

### 3.8. FT-IR Studies of chitin

The chitin was submitted to Infrared Spectroscopy, using Fourier Transform spectrophotometer (FT-IR), IFS 66 Bruker Mod (Chemistry Department, V.H.N.S.N. College, Virudhunagar, Virudhunagar district, Tamil Nadu, India). The chitin samples were characterized from 4000 to 400 cm-1 using infrared spectrophotometer (Shimadzu IR Prestige 21 FT-IR - ATR attached). The FT-IR spectrum of the extracted chitin sample was compared with that of the commercial chitin. The FT-IR spectrum for commercial chitin used in this study was taken from Thillai Natarajan et al. (2017).

## 4. RESULTS

Immobilised B. licheniformis reduced the protein associated with the shrimp shell powder by its production of extracellular protease in the medium. Fig.1 shows the level of extracellular protease enzyme produced by B. licheniformis (SSCL10). Extracellular protease production by the immobilised B. licheniformis was only 5 U/ml on the 2nd day of incubation and it increased to its maximal level of 16 U/ml on the 4th day of fermentation. Afterwards, protease activity was found to be decreased gradually (6 U/ml) on the7th days.

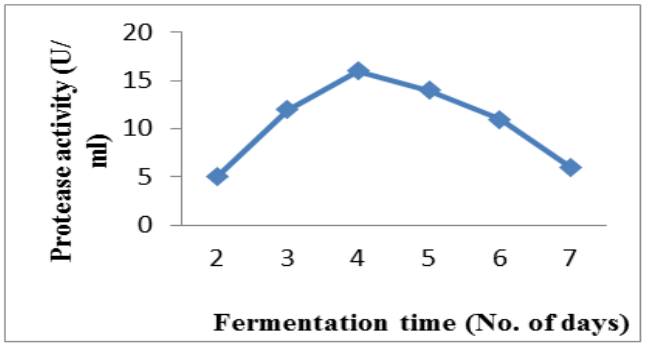


**Fig. 1: Protease production during deproteinisation of shrimp shell waste using Bacillus licheniformis (SSCL10)**

Fig. 2 shows the amount of protein content present in the shrimp shell powder during deproteinisation of B. licheniformis (SSCL10). On the 2nd day of incubation, protein content of the shrimp shell waste was 25% and it decreased to its minimal level on the 7th day of fermentation (3%). The protein content of the shrimp shell waste gradually decreased on the 7th day of incubation, due to the activity of protease enzyme.

 

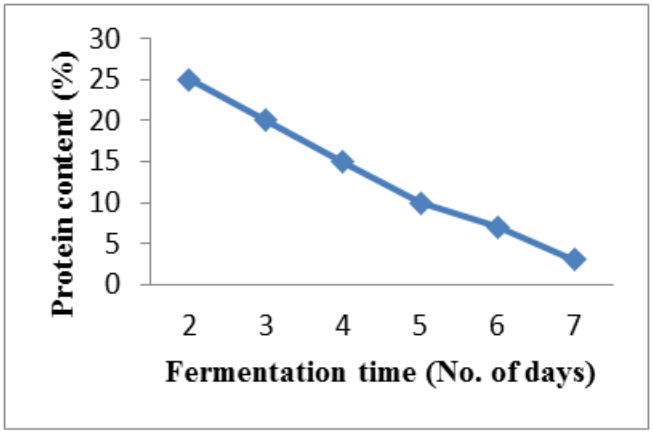


**Fig. 2: Percentage of protein content during deproteinisation of shrimp shell waste by Bacillus licheniformis (SSCL 10)**

The effect of deproteinisation efficiency (%) of immobilized B. licheniformis (SSCL10) upto 7 days of fermentation is presented in Fig. 3. The maximum of 90% deproteinisation efficiency of the shrimp shell waste powder was observed on the 7th day of incubation.

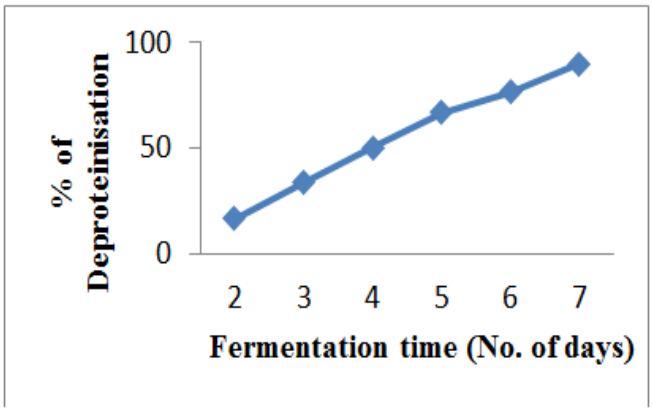


**Fig. 3: Effect of deproteinisation efficiency by Bacillus licheniformis (SSCL10)**

In the present study, Lactobacillus plantarum (MTCC 1407) was used for the process of deminerlization of shrimp shell waste for chitin extraction. Optimization conditions for chitin extraction showed that when 1% of Fe(NO3)3, as a mineral nitrogen source, was added to the fermentation medium, it resulted in a higher chitin yield (1.5 mg/g) when compared to the addition of yeast extract, peptone and ammonium chloride (as nitrogen source) which led to chitin yield of 0.8 mg/g, 0.6 mg/g and 0.4 mg/g respectively.

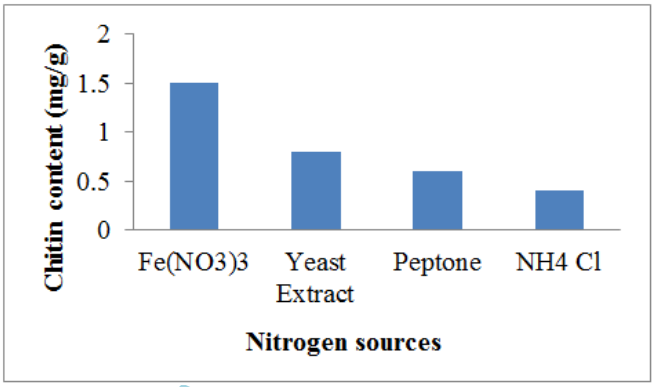


**Fig. 4 : Chitin extraction with Lactobacillus plantarum (MTCC1407) from shrimp shell waste with different nitrogen sources**

The deproteinated shrimp shell waste was mixed with various glucose concentrations of 5%, 10%, 15% and 20% for demineralisation and also inoculated with 10% inoculum of L. plantarium (v/w). After seven days of fermentation at 30oC, the demineralisation of shrimp shell waste was determined as amount of chitin. Results as shown in Fig. 5 indicated that 5% of glucose concentration, the chitin yield was 0.38 mg/g. But, 15% glucose concentration, it gave a higher chitin yield (1.6 mg/g) compared to others.

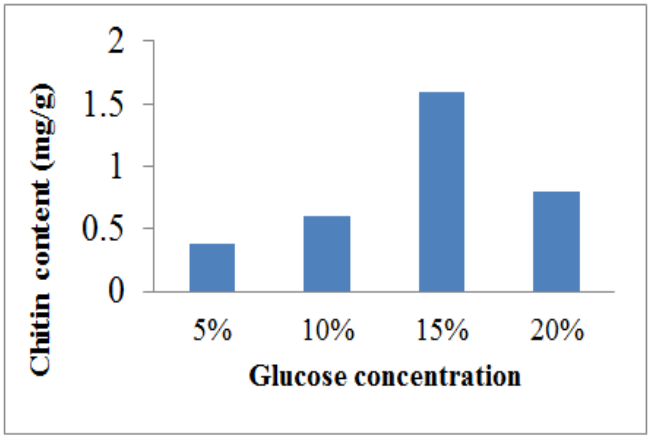


**Fig. 5 : Chitin extraction with Lactobacillus plantarum (MTCC1407) from shrimp shell waste with different glucose concentrations**

Fig.5 showed the increase in the amount of chitin from 0.38 mg/g to 1.6 mg/g in accordance with the increase in glucose concentration from 5% to 15%. These results were in accordance with pH changes and lactic acid production. These results ultimately indicated the effective demineralisation of shrimp shell waste powder. In the optimized medium (15% glucose), the utilization of glucose during demineralisation of the shrimp shell waste through lactic acid fermentation by L. plantarum (MTCC 1407) is presented in Fig. 6. About 94.7% of the sugar in the medium was utilized by the organisms on the sixth day (0.8 gm/100ml).

 

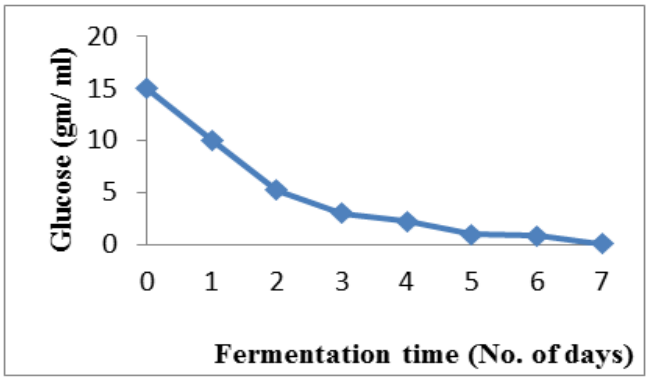


**Fig. 6 : Glucose consumption during demineralisation by Lactobacillus plantarum (MTCC 1407)**

The level of lactic acid produced by L. plantarum is shown in Fig. 7. Lactic acid concentration in the medium increased from 24 h of incubation (38 gm/l) to its maximal level (98 gm/l) on the 3rd day of fermentation.

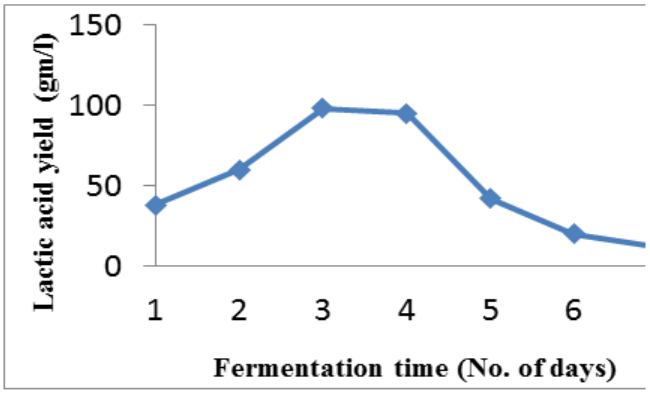


**Fig. 7 : Lactic acid production during demineralisation by Lactobacillus plantarum (MTCC 1407)**

The fast decrease in pH of the medium up to 3rd day (pH 3.4) coincided with increase in mineralisation related to chitin recovery. The chitin recovery rapidly increased after 3rd day of fermentation (13.12%) to 20.6% after 4th day of fermentation and 26.5% on the 5th day, 28.25% after 6th day of fermentation. On seventh day of fermentation, the chitin recovery was 28.8%. The process of chitin recovery continued till the end of the fermentation (7th day) as shown in Fig. 9.

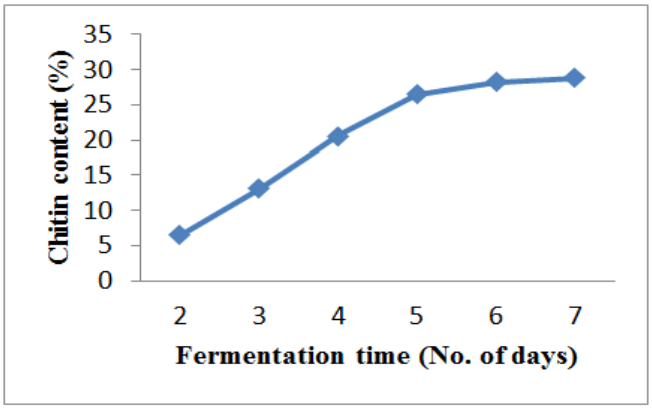


**Fig. 9 : Percentage of chitin content during deproteinisation of shrimp shell waste using Lactobacillus plantarum (MTCC 1407)**

The demineralisation efficiency (%) of Lactobacillus plantarum up to 7 days is presented in Fig.10. It was observed that maximum demineralisation of the shrimp shell waste had occurred on the 7th day of incubation (96%) from the initial substrate concentration.

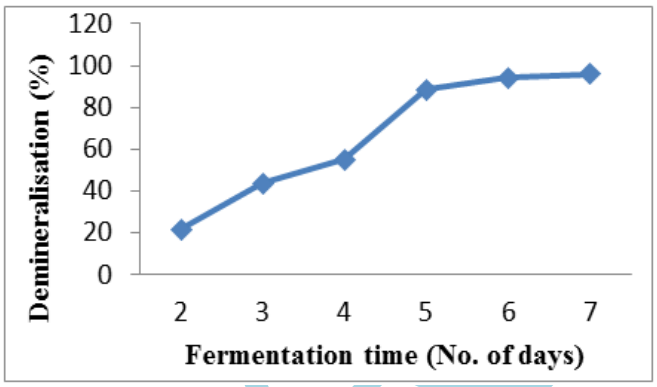


**Fig. 10 : Percentage of demineralisation of shrimp shell waste using Lactobacillus plantarum (MTCC 1407)**

After deproteinisation and demineralisation by B. licheniformis and L. plantarum, an appreciable amount (2.4 g) of chitin was extracted from 8 g of the shrimp shell waste powder with an efficiency of 30%. The effectiveness of lactic acid (produced by lactic acid bacteria) in removing the minerals from the shells and subsequent extraction of chitin have shown that lactic acid fermentation could provide an alternative to chemical treatment for extraction and recovery of chitin.In the present investigation, the moisture content of chitin was 5.4% and ash content was 0.86% on dry weight basis.

The FT-IR Spectrum of chitin prepared by B. licheniformis (SSCL10) and L. plantarum (MTCC1407) fermentation is shown in Fig. 12. The spectrum was compared with the commercial chitin from Thillai Natarajan et al., 2017 (Fig. 11). There are small impurities found on the chitin prepared from B. licheniformis and L. plantarum. But, it has a better percentage of same individual functional groups compared to commercial chitin.

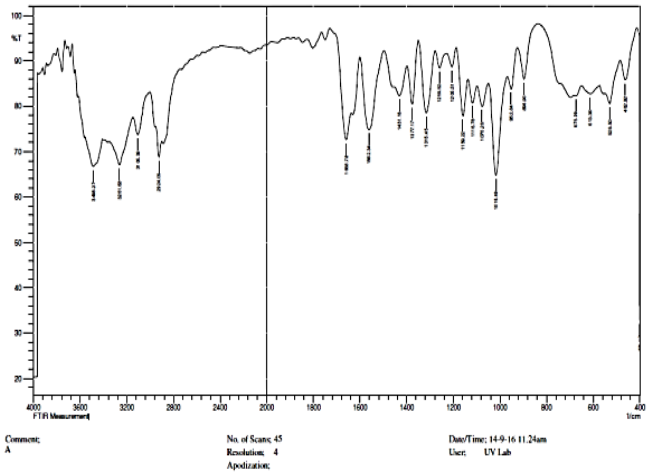

**Fig. 11 : FT-IR Spectrum of chitin prepared from deproteinisation of Bacillus licheniformis (SSCL10) and demineralisation of L. plantarum (MTCC1407)**

 

**Table 1. Functional groups of shrimp shell extracted chitin compared to a commercial standard chitin**

| S.No | Biological extracted of chitin from shrimp shell powder (Wave length in cm-1) | Standard chitin (Wavelength in cm-1) | Confirmation of the functional group | References |
|---|---|---|---|---|
| 1 | 3485 | 3448-3450 | OH stretching band | Thillai Natarajan *et al.*, 2017 |
| 2 | 3261 | 3300-3250 | NH stretching band of intra molecular H-bonding | |
| 3 | 3105 | 2891 | C-H stretching band of alkane | Thillai Natarajan *et al.*, 2017 |
| 4 | 2929 | | C-H stretching band of alkane | |
| 5 | 1659 | 1680-1660 | Stretching frequency of enol form of amide (amide I) | Duarte *et al.*, 2001 |
| 6 | 1562 | 1560-1530 | Stretching frequency of amide(amide II) | Duarte *et al.*, 2001; Ravindra *et al.*, 1998 |
| 7 | 1431 | 1419 | Asymmetrical deformation of $-CH_2$ group | |
| 8 | 1377 | 1340 | Asymmetrical deformation of $-CH_2$ group | |
| 9 | 1315 | | OH bending vibration | |
| 10 | 1259 | | C-O stretching of alcohol | Osada *et al.*, 2013 |
| 11 | 1205 | | C-O stretching of alcohol | Osada *et al.*, 2013 |
| 12 | 1159 | 1153-1156 | Antisymmetry stretching frequency of C-O-C bridge | |
| 13 | 1116 | | Bending vibration of C-C-C bridge | |
| 14 | 1076 | 1072 | Oligosaccarides stretching frequency of C-O-C bridge | |
| 15 | 1016 | | C-N stretching of | |
| 16 | 952 | 952 | OH deformation stretching frequency in dimer(amide III) | Thillai Natarajan *et al.*, 2017 |
| 17 | 897 | | Stretching frequency of glycosidic linkage | |
| 18 | 675 | | C-H out- of-plane bending vibration | |
| 19 | 613 | | NH stretching | |

As shown in the FT-IR spectrum of recovered chitin derived from the shrimp shell (Fig.11), the bands at 3485 cm-1 are attributed to the OH stretching. The shape and intensity of these peaks will change if the hydrogen bonding network in chitin is altered. The bands ranging from 2824 to 3105 cm-1 represent C-H stretching bands of alkane. And the CH bending, symmetric CH3 deformation and CH2 wagging bands appear at 1431 and 1377 cm-1. The peaks at 1658 and 1562 cm-1 are assigned to Amide I band (two types of hydrogen bonds in a C=O group with the NH group of the adjacent chain and the OH group of the inter chain). Amide II band (in-plane N–H bending and C–N stretching mode) was observed at 1562 cm-1, respectively. The bands ranging from 1027 to 1163 cm-1 are attributed to the asymmetric bridge oxygen and C–O stretching (Osada et al., 2013). The shrimp chitin showed an intense peak ranging from 1259 to 1205 cm-1 are attributed to the C–O stretching The shrimp chitin showed an intense peak at 1562 cm-1 which corresponded to the N–H deformation of amide II . The bands at 1659 cm-1 are attributed to the vibrations of the amide I band. These bands can be clearly observed in shrimp shell extracted chitin. The sharp band at 1377 cm-1 corresponds to a symmetrical deformation of the CH3 group. The interpretation of FTIR analysis of the samples is compared with interpretation is the FTIR of standard chitin for the possible presence of functional groups.

## DISCUSSION

 

Commercial process for chitin extraction from shrimp shell waste is mainly based on chemical method where the shell is deproteinised by alkali (NaOH) treatment and demineralized by inorganic acid (HCl) treatment. This method predominantly denatures the chitin and makes them less usable for its biological functions(Shahidi et al., 1999). Moreover, the use of alkali, acids and organic solvents can be toxic, corrosive, degradative and mutagenic which makes the extracted chitin unsuitable for medicinal use. Waldeck et al. (2006) reported that strong acid and alkali treatment are used at high temperatures to remove minerals and proteins. These chemicals have been reported to cause depolymerisation of the chitin and affect its molecular weight and viscosity after solubilisation. Percot et al. (2003.) reported that using inorganic acid such as HCl for the demineralisation of chitin, results in detrimental effects of the purified chitin.

Biological method of chitin production by using organic acids producing bacteria and protease producing bacteria reduces the sources of environmental pollution as well as depolymerisation of chitin (Healy et al., 2003). Lactic acid bacterial fermentation for demineralisation has been occasionally reported for shrimp waste (Shirai et al., 2001) and prawn waste (Shirai et al., 1998).

The employment of microorganisms or proteolytic enzymes for deproteinisation of marine crustacean wastes is a current research trend to convert wastes into useful bioactive substances. It is a simple and environment friendly alternative to chemical methods employed in the extraction of chitin.In this study, the local isolate Bacillus licheniformis (SSCL10) was used to deproteinise shrimp shell powder due to its high protease producing ability. The maximum of 90% deproteinisation efficiency was obtained after seven days of incubation of B. licheniformis. Bustos and Muchael (1994) have compared the effects of microbial and enzymatic deproteinisation and found a maximum value of 82% deproteinisation was achieved with P. maltophila after seven days of incubation.

In the present study, the effect of Lactobacillus plantarum (MTCC 1407), a lactic acid producing bacteria, in demineralisation of shrimp shell wastes for chitin extraction was studied. Biological demineralisation has also been reported for chitin production from crustacean shells by microbial process involving species like L. pentosus 4023 (Bautistat et al., 2001) or by a natural probiotic (milk curd) (Prameela et al., 2010). According to Rao et al. (2000), pH after fermentation was lower at higher concentration of glucose since lactic acid is produced through break down of glucose. The deproteinisation was however inversely proportional to glucose concentration, which decreased almost four fold from 45.72% to 13.81%.

Similarly in the present work, glucose concentration at 15% resulted in a higher chitin yield (1.6 mg/g) and was more or less substantiated by Rao et al. (2000). Also, the proportions of the additional starter and glucose were important for the lactic acid bacterial fermentation to demineralize the raw shell wastes (Shirai et al., 2001; Rao et al., 2002). In our study, glucose was chosen for it being a readily fermentable sugar. 15% of glucose was used as optimal concentration for lactic acid fermentation. The glucose level of 15% used for demineralisation by lactic acid fermentation was able to produce enough lactic acid and reduce the pH of the medium as reported in earlier studies (Zakaria et al., 1998; Cira et al., 2002).

The results of this work, extraction of chitin from the shrimp shell waste using deproteinisation was the first process was done by local soil isolate Bacillus licheniformis (SSCL10) and then demineralization was carried out by Lactobacillus plantarum (MTCC 1407). Wahyuntari et al. (2011) compared the order of microbiological deproteinisation and demineralisation in chitin extraction of shrimp shell waste. Deproteinisation process by using Bacillus licheniformis strain JS followed by demineralisation of shrimp waste by Lactobacillus acidophilus produced higher chitin extraction efficiency than demineralisation process followed by deproteinisation. On contrary, Duan et al. (2012) reported an improvement in the removal of minerals using the Lactobacillus acidophilus (SW01), with more protein removed. Protein removal exposed more minerals to the acidic environment, which has led to the improvement in the removal of minerals.

Infrared (IR) spectroscopy is one of the widely used analytical techniques available to scientists working on chitin. It is based on the vibrations of the atoms of a molecule. The energy of each peak in an absorption spectrum corresponds to the frequency of the vibration of a molecular part, thus allowing qualitative identification of certain bond types in the sample. An IR spectrometer usually records the energy of the electromagnetic radiation that is transmitted through a sample as a function of the wave number or frequency. Due to the above mentioned reasons the analytical capitalization of infrared spectrums was started. Figures 4 shows the infrared spectrum of the chitin in the spectrum field 4000 – 400 cm–1, in the form transmittance vs. wave number (–symmetric stretch; –asymmetric stretch; δ–deformation; ω–wagging). The bands are generally large due to the macromolecular character of the compound and because of the numerous intermolecular bindings of hydrogen, manifested even in the solid state of the sample (Barbat et al.,2013).

## CONCLUSION

The shrimp shell waste was deproteinised using immobilised Bacillus licheniformis (SSCL10) by submerged fermentation. Maximum level of extracellular protease (16 U/ml) was produced on the 4th day of fermentation. The protein content of shrimp shell was greatly reduced to 3% at the end of fermentation (after 7 days). Maximum deproteinisation of B. licheniformis was 90%. Lactobacillus plantarum (MTCC 1407) was used for the process of demineralisation for chitin extraction. The maximum demineralisation of 96% was observed on the 7th day of fermentation. The chitin extraction from shrimp shell after deproteinisation and demineralisation showed 30% efficiency. The extracted chitin was confirmed by comparing it with the FT-IR spectrum of commercial

 

chitin. It is concluded that the chitin extracted in the present study is an effective one and it is a new method based on the use of proteolytic bacteria and lactic acid bacteria employed for deproteinisation and demineralisation processes. This method allows producing a good quality chitin. The conventional demineralisation, deproteinisation and decoloration method of chitin extraction from crustacean waste is costly and use of chemicals causes environmental problems. The bioconversion of shrimp shell waste into a useful product has been proposed in the present study as a waste management alternative to the disposal of shellfish wastes in the environment.

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