ABSTRACT

Biodegradation of tiger prawn waste was carried out using *Lactobacillus plantarum* (NCIM 2912) and *Lactobacillus casei* (NCIM 9595). *Lactobacillus plantarum* was found to be more efficient in term of % degradation. Detail investigation on the influences of key parameters such as glucose concentration, temperature etc. on biodegradation was done to study the efficiency of *Lactobacillus plantarum* for demineralization of tiger prawn shell waste. The fermentation experiments have been carried out using 150, 200, 250 and 300 micron particle size of shell waste and 8% inoculation as the starter culture upto 5 days. Various concentrations of (0, 2, 4 and 8%) of glucose were supplemented as initial source of carbon for the microorganism. On the 5th day of fermentation the pH for varying glucose level (0, 2, 4 and 8%) was found to be 7.3, 6.9, 6.1 and 5.1. The corresponding Total Titratable Acidity (TTA) was observed as 0.67, 1.12, 1.7 and 6.2%. Maximum demineralization of 80% was achieved with particle size of 150 micron, at 8% glucose, 8% inoculum and 30°C. Correlation between pH and % Demineralization gave a negative relationship ($R^2=0.573$) and a positive relation between pH and % demineralization ($R^2=0.860$).

Key Words: Tiger prawn shell waste, Biodegradation, *Lactobacillus plantarum, Lactobacillus casei*, Lactic acid fermentation, Demineralization, Biological waste treatment

INTRODUCTION

A huge quantity of sea food waste is generated during the processing of various sea foods worldwide. This huge amount of waste residue is either discharged to landfills or various dumping sites without any pretreatment where they lie for years. The dumping of this waste causes severe organic pollution and as a result physical disturbance of hydrological regimes including the conversion and degradation of coastal ecosystem occurs. In order to degrade this shell waste, chemical degradation using concentrated acids such as HCl, HNO₃, H₂SO₄, CH₃COOH and HCOOH have been tried by several researchers. However, the chemical methods are expensive and detrimental to the environment.

From the literature, it is evident that the limitations of the chemical method for the degradation of sea food can be largely overcome by the biological method of demineralization and hence research interest has been shown in recent years in this direction. Lactic acid fermentation of crustaceans shell waste has been reported to be studied as a potential biological method of degradation. India is one of the major tiger prawn producing and processing country in the world. India is the second largest contributor of the net Finfish and Shellfish produced globally in the world. India produces more than 115 000 tonnes of tiger prawn each year and since 40% of it discarded as waste then nearly 46000 tonnes of prawn waste is produced each year. The lactic acid fermentation may be an effective method for the degradation of this waste.

AIMS AND OBJECTIVES

To study the fermentation of tiger prawn waste using lactic acid bacteria and to investigate the effects of various parameters such as % glucose, temperature and particle size, solid to liquid ratio
which will facilitate the demineralization process giving high percentage of degradation.

MATERIAL AND METHODS

Preparation of tiger prawn shell
The tiger prawn shell wastes were procured from local market of Paradeep, Orissa India. Waste Tiger Prawn shells upon receipt, is washed with tap water to remove flesh residues and other contaminants. It is then dried in oven at 60°C for 24hrs. The dried shell is grinded through a grinding machine (Bajaj Model # DR5000) and subjected to mechanical sieve separator to get different particle size of 150,200,250 and 300micron. Dried ground shell is kept in opaque air tight glass bottles and stored at ambient temperature until used.

Micro-organisms
The strain of Lactobacillus plantarum (NCIM 2912) and Lactobacillus Casei (NCIM 2586) were obtained from NCIM Pune, in form of slant and was subcultured from time to time for their maintenance.

Media
MRS agar (HiMedia) having the composition of Peptone 10g/l, Beef extract 10g/l, Yeast extract 5.0g/l, Dextrose 20g/l, Polysorbate-80 1.0g/l, Ammonium citrate 2g/l, Sodium acetate 5g/l, Magnesium sulphate 0.1g/l, Manganese sulphate 0.05g/l, Dipotassium phosphate 2g/l and Agar 12g/l and pH 6.5 is used as growth media. The solid media is mixed with 100 ml distilled water. The media is then poured in the petri-plates and allowed the agar to solidify. A loop full of microbial colonies are taken from the slant and inoculated in the petri plates. The petri plates are then kept in the incubator at 35°C for the growth of the culture and stored at 4°C.

Preparation of inoculum
In order to prepare a liquid culture, cells are transferred from the petri plates into 100 ml of sterile Man Rogosa Sharpe (MRS) broth and incubated at 35°C for 24 hrs. 1ml of the starter prepare 1% inoculation and incubated with shaking (120 rpm) at 35°C for 24 hr culture is then transferred into 100ml of sterile Man Rogosa Sharpe (MRS) broth.

Cell density
To know the biomass of the inoculum the cells are harvested by centrifuging (C31 Cooling centrifuge, Remi-India, India) at 10000 for 10 min. The harvested cells are washed with 0.1% saline water and serially diluted with 0.1% (v/v) NaCl solution by adding sterile distilled water. After suitable dilution, 0.5ml of it was spread plated onto MRS agar and the colonies were counted by colony counter.

Fermentation
5gms of prawn waste with 10% moisture content is mixed with 50ml of glucose solution (concentration 0, 2, 4 and 8% w/v), 200 ml MRS Broth inoculated with 8% (v/v) inoculum in a clean 500 ml conical flask. The flasks are then sealed with parafilm and the mixture is fermented for 5days in an orbital shaking incubator at 120 rpm and 35°C. After the fermentation, the sample of each batch of experiment is filtered by Whatman filter paper to separate the liquid and solid. The filtrate is used for analysis of pH and TTA. The solid residue is subjected to repeated wash with Milli-Q water (1:10, w/v) and used for further analysis of moisture and ash content.

Analysis

Dry weight measurement and ash content
Dry weight of the solid residue is measured after drying at 60 °C for 48 h in a hot air oven until a constant weight is achieved. Ash content (Relative Residual Ash; RRA) is determined by combustion of dried sample at 600°C for 6hrs in a muffle furnace.

pH and Total Titratable Acidity (TTA)
The filtrate obtained by filtration of the fermentation broth was used for the determination of pH and TTA. The pH of the filtrate is measured with a pH meter (Beckman, USA). The Total Titratable Acidity (TTA) is determined in diluted sample by titration with 0.1 M NaOH required to increase pH to 8.0.

SEM Analysis
The elemental composition of the prawn shell waste before and after biodeminerlisation were determined by Scanning Electron Microscopy (SEM, model No. JEOL JSM-6480 LV, Japan) using the elemental analyzer. The surface morphological studies have been done by Scanning Electron Microscopic analysis.
RESULTS AND DISCUSSION

Elemental analysis
The total mineral content of the prawn shells used in this study was 30.32%. This value is within the range of 30-50% as reported for shrimp by. The most abundant minerals in the shrimp shells were Ca, P, Si, Al, S and Na which accounted for 41.75, 8.06, 2.76, 2.41, 1.08 and 0.74% of the total shell mineral composition, respectively. Calcium was thus identified to be present in much higher amounts than other minerals. Similarly other researchers also stated that the major mineral component of shellfish waste is calcium. Others reported that the most abundant minerals in prawn shell were Ca, Mg, Na, Sr, K and Fe and that calcium was the most abundant amongst all. These conditions were well in agreement with the elemental analysis results achieved.

Effect of types of Lactobacillus bacteria on biodemineralization
The fermentation experiments have been carried out using Lactobacillus plantarum and Lactobacillus casei to see their efficiency towards the demineralization of tiger prawn shell. The fermentation conditions were maintained as 35°C, 120rpm, 5 days of degradation time and initial pH of 8. The experimental result obtained during the 5 days of fermentation is presented in Table 1. It is observed that L. plantarum shows higher efficiency than the L. casei in terms of % demineralization. The % demineralization of 79.51 and 76.22 were achieved with L. plantarum and L. casei respectively. It has also been observed that L. plantarum could tolerate much lower pH and acidic conditions. The observation of high acidic tolerance of L. plantarum has also been reported. Due to high demineralization efficiency, L. plantarum was used for detail investigation for the biodegradation of prawn waste.

Effect of fermentation parameters
The biodemineralization of tiger prawn shell using lactic acid fermentation was carried out at different concentration of glucose (0, 2, 4 and 8%) and particle size (150, 200, 250 and 300 micron). Table 2 shows the changes in pH, TTA and RRA with the variation in particle size and glucose concentration.

Effect of glucose supplement on pH, TTA and demineralization
Effect of glucose on pH
As shown in Fig. 1, the pH for 2% and 4% glucose decreased drastically from pH 8 to pH 6.4 and 6.2 respectively in the first day of fermentation. A slight increase in pH of pH 7.1 and 6.5 respectively was observed till second day and then it remains constant for the next three days of fermentation. Whereas, the pH with 8% glucose was observed to be decreased continuously up to 3 days from an initial pH 8 on the first day to a pH 4.8 on the third day. The pH observed with 0%, 2%, 4% and 8% glucose after five days of fermentation on an average were 7.36, 6.9, 6.1 and 5.1 respectively.

Effect of glucose on %TTA and % RRA
The TTA is highly dependent on the glucose concentration which is evident from this study. At 30°C, 150 micron particle size and 5 days of fermentation, the TTA was found to be 0.9, 1.2 and 3.0% with 2, 4 and 8% glucose respectively. The TTA values with 2 and 4% glucose concentration were shown to be increased drastically on the first day and then slowly decreased. When 8% glucose was used the TTA was found to increase gradually for three days and then there was no change in TTA. The Relative Residual Ash (RRA) on the other hand decreased with an increase in TTA as shown in Table 2. The RRA reduced rapidly during the first day for 2 and 4% glucose concentration and it was found to be 56.57 and 47.7% respectively. The RRA for 2% and 4% glucose supplement was found to be 53.4 and 40.2 respectively and the value was found to be 20.1% with 8% glucose concentration.
Table 1: Cell growth, pH, TTA and RRA in liquid broth after 5 days

<table>
<thead>
<tr>
<th>Name of the microbes</th>
<th>pH</th>
<th>TTA</th>
<th>Cell Growth (cfu/ml)</th>
<th>% demineralization after 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 24</td>
<td>48 72 96 120</td>
<td>24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td>L. plantarum</td>
<td>8.0 5.9 5.1 4.8 4.9 5.2</td>
<td>0.9 1.4 4.4 6.1 7.1</td>
<td>20.9×10^6 79.51</td>
<td></td>
</tr>
<tr>
<td>L. casei</td>
<td>8.0 6.1 5.5 4.9 5.1 5.4</td>
<td>0.8 1.1 3.6 5.8 5.9</td>
<td>20.3×10^6 76.22</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1: The effect of %glucose and demineralization time on pH variation

Table 2: pH, TTA, RRA for different particle size and glucose level after five days of fermentation

<table>
<thead>
<tr>
<th>Factors</th>
<th>pH</th>
<th>TTA (%)</th>
<th>RRA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (µ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.76</td>
<td>0.6</td>
<td>55.9</td>
</tr>
<tr>
<td>2</td>
<td>6.9</td>
<td>0.9</td>
<td>53.4</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>1.2</td>
<td>40.2</td>
</tr>
<tr>
<td>8</td>
<td>5.1</td>
<td>7.1</td>
<td>20.1</td>
</tr>
<tr>
<td>2</td>
<td>7.1</td>
<td>0.71</td>
<td>55.7</td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>2.2</td>
<td>40.6</td>
</tr>
<tr>
<td>8</td>
<td>5.3</td>
<td>7.4</td>
<td>20.3</td>
</tr>
<tr>
<td>2</td>
<td>7.2</td>
<td>1.4</td>
<td>48.5</td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>2.4</td>
<td>40.9</td>
</tr>
<tr>
<td>8</td>
<td>5.6</td>
<td>7.8</td>
<td>20.7</td>
</tr>
<tr>
<td>2</td>
<td>7.84</td>
<td>0.87</td>
<td>55.8</td>
</tr>
<tr>
<td>4</td>
<td>7.5</td>
<td>1.5</td>
<td>49.8</td>
</tr>
<tr>
<td>8</td>
<td>5.6</td>
<td>8</td>
<td>21.3</td>
</tr>
</tbody>
</table>
Effect of glucose on demineralization
The concentration of glucose was observed to have a great impact on demineralization of tiger prawn waste as shown in Fig. 3. For glucose concentration of 0%, 2% and 4% the % demineralization rapidly increased on the first day and then remained almost constant. Whereas, the % demineralization for 8% glucose was found to increase till the third day and then became almost constant. Thus as shown in Fig. 3, the % demineralization increased with an increase in the glucose concentration and maximum % demineralization of 79.99% was achieved with a glucose supplementation of 8 % after 5 days.

Effect of particle size on demineralization
The effect of particle size of prawn waste on bio demineralization after five days of fermentation was investigated using waste of 150, 200, 250 and 300 micron size. The experimental results are shown in Fig. 4. From the experimental results, it is evident that the demineralization (%) increased with the decrease in particle size. Rate of demineralization was observed to be higher using medium size particles compared to the rate obtained with waste of larger particle size. Though there is no remarkable difference in % demineralization was observed with medium and small particle size but the highest demineralization of 79.77% was achieved by the use of smaller particle size of 150 micron. At day 5, the demineralization (%) with150, 200, 250 and 300 micron particle size were obtained as 79.9%, 79.6%, 79.3% and 78.7%, respectively. Thus amount of metal ions leached from 300 micron particles is same as that for 150 micron particles. Moreover the volumes of 300 micron particles are eight times to ones of 150 micron particles therefore for convenient waste minimization consideration, it is more economical to produce 300 micron particles.
Effect of temperature on demineralization

The fermentation experiments have been carried out at varying temperature in the range of 25°C to 40°C to investigate the effect of temperature on demineralization (%). Both glucose concentration and inoculums was maintained as 8%. From the results shown in Fig. 5, the demineralization (%) is shown to be increased with increase in temperature from 25°C to 30°C and then the activity of the microbe was shown to be decreased with further increase in temperature. The maximum demineralization (%) of about 80% was observed at the temperature of 30°C followed by 78.68% demineralization obtained at 35°C.

![Fig. 5: Effect of temperature on % demineralization](image)

Effect of solid to liquid ratio

An attempt has also been made to study the effect of the solid to liquid ratio on demineralization. For this the fermentation experiments have been performed using different solid to liquid ratio of (5, 7.5, 10, 20 and 30%) prawn shell waste with 8% inoculums and 8% glucose solution. The fermentation experiments were conducted at 30°C and the particle size of the waste was 150 micron. It is observed that demineralization efficiency decreases with the increase with the solid to liquid ratio as shown in Fig. 6. The maximum % demineralization of 85.68% was achieved with 5% prawn shell waste followed by 80% demineralization obtained with 7.5% and 71% demineralization with 10% respectively. Thus 10% solid to liquid ratio is found to be most favorable for demineralization of prawn shell by lactic acid fermentation. Relation between pH and TTA with Demineralization% was analyzed and summarized. There is a strong correlation between pH and demineralization % and TTA and demineralization%. The result of correlation is shown in Fig. 7(a) and Fig. 7(b). In this study, the co-relation between pH and
Demineralization % is \( R^2 = 0.860 \) which is in good agreement with the result reported by Jung et al. for demineralization of red shell waste by lactic acid fermentation.\(^{13}\) The correlation is found to be \( R^2 = 0.573 \), whereas a correlation between pH and demineralization level is \( R^2 = 0.860 \) reported by Jung et al.\(^{13}\)

![Graph showing demineralization % vs. solid to liquid ratio](image1)

**Fig. 6**: Effects of solid to liquid ratio on demineralization % after five days of fermentation.

![Graph showing pH vs. demineralization %](image2)

**Fig. 7(a)**: Correlation between pH and demineralization (%) during lactic acid fermentation with prawn shell waste.

![Graph showing TTA vs. demineralization %](image3)

**Fig. 7(b)**: Correlation between TTA and demineralization (%) during lactic acid fermentation with prawn shell waste.
Topographical study

A topographical study of the prawn shell biowaste was also carried out before and after fermentation to study the change in surface characteristics of the waste. It was observed that before fermentation the prawn shell gave a coarse look as can be seen in the Scanning electron micrograph (Fig. 8). A highly porous and fibrous substance was obtained after fermentation which indicates that the cementing material mainly calcium carbonate along with other minerals covering the prawn shell have been removed successfully. This highly porous organic substance is the chitin biopolymer which has come out of the cementing material. Chitin is a widely used in the food industry, medicinal fields, chemical industries, textiles, water treatment plants, etc.

The biodemineralization of prawn shell waste was investigated by lactic acid fermentation under varying condition using lactic acid bacterium L. plantarum and L. casei. Out of the two microbial strains Lactobacillus plantarum has shown better efficiency than L. casei towards the demineralization of prawn waste. The demineralization was observed to be highly depended on the glucose supplement. The % demineralization increased with an increase in the glucose concentration. However, the maximum % demineralization of 79.99% was achieved with a glucose supplementation of 8% which is higher than the value (76%) obtained using L. Casei. The detail investigation was carried out on the biodegradation of prawn shell using L. plantarum under varying condition of glucose concentrations, particle size and solid to liquid ratio to establish the optimum biodegradation condition.

The result obtained under present study are comparable with the results reported on the biodegradation of other sea food waste by lactic acid fermentation using various types of lactic acid bacterial strain. Some researchers reported fermentation efficiency in terms of demineralization of 72.5±1.5% using P. acidolactici CFR2182 and shrimp waste.14,15 Rao and Stevens 2000 achieved a demineralization of 81% with
L. plantarum and shrimp waste by adding glacial acetic acid before fermentation. However, in the present study even without adjustment of the initial pH there was no spoilage noticed. The pH decreased rapidly from pH 8 to pH 6 for the glucose levels of 2% and 4% for the first day. The pH dropped from pH 8 to pH 5.4 with 8% glucose concentration and at the inoculums level of 8%. In all cases the acidification was fast enough to avoid any kind of spoilage in the liquid broth or the prawn waste during fermentation. On the third day, the pH further dropped to pH 4.9 for 8% glucose (Fig. 1). Low pH as observed in this study is in conformity with many other such studies 12, 15. Thus, under proper fermentation conditions and with sufficient 8% glucose concentration and 8% inoculums a minimum pH of 4.9 and TTA of 6.2 was achieved. TTA is considered as an important parameter in the study because acidification of prawn shell biowaste prevents the growth of spoilage microorganisms. Similar conditions where the production of organic acids by the lactic acid bacterium L. plantarum decreased the pH and made the environment suitable against spoilage microorganisms were also reported by other researchers.  

Particle size of the prawn biowaste has shown to have an impact on the rate of demineralisation. The demineralization gradually increased with the decrease in particle size. As shown in the Fig. 3 at day 5, the %demineralization of 150, 200, 250 and 300 micron waste was 79.9%, 79.6% 79.3% and 78.7% respectively. Thus highest demineralization was achieved for 150 micron size and the least for the smallest particle size 300 micron. According to some researchers, larger particle sizes require longer swelling time resulting in a slower deacetylation rate. Similarly, degradation rate might have been slowed down due to larger particle size. An attempt was also made to find out the effect of the solid to liquid ratio on demineralization under the optimum demineralization parameters. It was found that, demineralization efficiency decreases with an increase in the solid to liquid ratio. Demineralization % over 20% amounts of prawn shell waste were much lower compared with those of 5% and 10% of prawn shell waste, mainly because of lower acid to waste ratio. Hence, amount of lactic acid produced by the lactobacillus should be enough for demineralization of the shells. Another reason for poor demineralization might be that an increase of solid concentration over 20 % increases slurry viscosity and decreased rate of mass transfer, which results in poor yield of formation of calcium lactate. The best efficiency in demineralization was achieved with 5% Prawn shell waste. Similar results were also reported by other researchers. The effect of temperature on fermentation and hence biodegradation was also investigated. It was found that the % demineralization increased as the temperature increased from 25 to 30°C but % demineralization decreased again when the temperature was increased further. A maximum of 80% demineralization was achieved with a temperature of 30°C which decreased to 78.68% and 78.38% when the temperature was increased to 35°C and 40°C respectively. Similar result of maximum demineralization at 30°C was obtained other researchers when red crab shell was used.  

CONCLUSION  

Topographical analysis using SEM proved that once the cementing minerals are removed from the surface of the biowaste it becomes a highly porous and fibrous organic material as can be seen in image B and C respectively. This substance is known as chitin. Chitin is a biodegradable and biocompatible natural polymer and has immense application in almost all kinds of industries (e.g. water treatment, pulp and paper industry, biomedical devices and therapies, cosmetics, biotechnology, agriculture, food science and membrane technology).  

REFERENCES  

3. Bautisa J., Cremades O. and Corpas R., Preparation of chitin by acetic acid


