

BIOREMEDIATION OF SHRIMP BIOWASTE BY USING NATURAL PROBIOTIC FOR CHITIN AND CAROTENOID PRODUCTION AN ALTERNATIVE METHOD TO HAZARDOUS CHEMICAL METHOD

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ABSTRACT: The production of chitin and carotenoids from shrimp biowaste by using natural probiotic has been studied. The fermentation studies were determined by varying inoculum levels from 1 to 10%, glucose concentration from 0 to 15% and incubation time 0 to 72 hours. The maximum decrease of pH 3.01 and an increase of total titrable acidity (TTA) 2.715 were obtained for incubation period of 72hours with 5% inoculum level and 15% glucose concentration. The effect of fermentation by natural probiotic on the production of chitin (as indicated by deproteinization and demineralization efficiency) and recovery of carotenoids were also studied. Deproteinization of 89% and demineralization of 69% were obtained by fermentation of shrimp biowaste with natural probiotic. The carotenoid and chitin recovery from shrimp biowaste was 72.6% and 5.65% respectively. As the process by using natural probiotic was found to be efficient, economical, it becomes an alternative method for hazardous chemical method used in extraction of chitin and carotenoids.

Key words: Shrimp biowaste, Probiotic, Deproteinization, Demineralization, Chitin, Carotenoid.

INTRODUCTION:

Excess toxic chemicals such as HCl, Acetic acid and NaOH are introduced into aquatic ecosystem as by-products of shrimp processing industries are spoiling the aquatic flora and fauna. They are highly toxic as they are easily soluble in water and they may be rapidly absorbed into living systems. Shrimp processing is the one of the largest industrial fish waste in the country which produces more than 1, 00,000 tonnes of industrial wastes (Mathew and Nair, 2006). These marine industries generate large quantities of solid waste in the form of head and body carapace. These body parts comprise 48-56% depending on the species (Sachindra, *et al.*, 2005). Shrimp waste is one of the important sources of chitin and natural carotenoids. Shrimp biowaste being alkaline (pH 7.5-8.0) supports the growth of undesirable putrefying micro flora resulting in spoilage (Rao and Stevens, 2005; Hall, 1994).

Treatment of this biowaste using hazardous chemicals involves alkalis (usually 4% NaOH) for deproteinization and strong acids (4% HCl and acetic acid) for demineralization, making this process ecologically aggressive and is a source of pollution (Rao and Munoz, 2000). While the chemical process isolates chitin efficiently, the protein and carotenoids components are rendered useless during protein removal and demineralization stages (Pasquel and Babbil, 1991). Although the shrimp biowaste is usually dried on beaches it encourages not only environmental pollution but also reduces removable components in this biowaste. Chitin is a versatile, environmentally friendly modern material isolated from shrimp biowaste. It has a wide range of applications in areas such as medicines, fine chemicals for water treatment, pulp and paper industries, biomedical devices, therapies, cosmetics, membrane technology, biotechnology, food applications and textiles (Nesreen Samir Mohmoud and Abdelkader cehaly, 2008; Park, *et al.*, 2005; Boutistat, *et al.*, 2001; Yang, *et al.*, 2000).

The traditional chemical method creates a disposable problem due to large amounts of toxic waste without further treatment would pollute the environment. As regulations have become stricter now, there is a need to treat and utilize the waste in most efficient manner. Therefore there is a significant interest regarding recycling of shrimp biowaste. Hence the conversion of shrimp biowaste into ensilage advantageously upgrades the biowaste with this approach being eco friendly safe, technologically flexible and economically viable.

An alternative method, using fermentation by microorganisms has been emerged, which can do considerable extent replacing the expensive and non environmental friendly chemical process (Wang and Chio, 1998; Rao MS, *et al.*, 2001; Rao MS, *et al.*, 2002; Longo and Combes, 1995). Micro organisms studied include *Lactobacillus plantarum*(Rao MS, *et al.*, 2000), *Pseudomonas aeruginosa* (Wang and Chio, 1998), *P. maltophilia*(Wang and Chio, 1998), *Bacillus subtilis* (Yang, *et al.*, 2000; He H, *et al.*, 2006), *Lactobacillus paracasei* (Shirai K, *et al.*, 2001), *Lecanicillum fungicola*(Laura RC, *et al.*, 2006) , *Pencillium chrysogenum* (Patidar P, *et al.*, 2005), *Pediococcus acidilactici* (N.Bhasker , *et al.*, 2007). Fermentation of shrimp head biowaste using these microorganisms resulted in medium conditioning, supposedly by production of lactic acid and proteases. Lactic acid produced by breakdown of glucose creates low pH condition of ensilation that suppresses the growth of the spoilage microorganisms (Cira LA, *et al.*, 2002; Jung, *et al.*, 2005). 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 first objective of the present work is to ascertain the effect of inoculums supplement, glucose supplement on efficiency on demineralization (DM) and deproteinization (DP). Secondly, the artificial media used to cultivate various microorganisms involved in fermentation process is MRS (de Man, Rogosa and Sharpe) agar medium, which is expensive and hence the alternative source to it is natural probiotic. It increases economic efficiency of the process. The usage of hazardous chemicals in extraction of chitin from shrimp biowaste can be prevented. In this work the bioremediation of shrimp biowaste by using natural probiotic was studied with the effect of pH, TTA, inoculums and sugar levels etc. Finally the effective fermentation on deproteinization and demineralization, carotenoid recovery and chitin production were studied.

MATERIALS AND METHODS

Materials:

Shrimp waste from *Penaeus monodon* comprising of head was obtained from Shrimp processing industries located at Bhimavaram, West Godavari District, Visakhapatnam, Andhra Pradesh, India. It was transported to the lab under frozen conditions and kept at -20°C till further use. The material was thawed in running water before use.

Preparation of inoculum

The natural probiotic (milk curd) was prepared by heating two quarters of milk to a temperature range of 180 to 185 °F. 1ml of fresh curd was taken and inoculated to 100ml of milk at 40°C. The curd was made according to E. Tobia method (E. Tobia , 1982).

Preparation of shrimp waste

The frozen (-20°C) shrimp was thawed overnight in a refrigerator (4°C) and washed with boiling distilled water which can remove endogenous microbes from the shrimp. Then the shrimp was homogenized by using local made electrical mill.

Effect of fermentation on recovery of chitin and carotenoids

Homogenized shrimp biowaste (100gm) was mixed with 100ml of distilled water. To this different inoculum levels (1%, 5%, 10%), different glucose concentrations (0, 2.5, 5, 10, 15 gm) and 2% NaCl were added and fermented for 72hrs in 250ml flasks. The experiment was carried out in three different batches. Each sample was filtered by using cheese cloth to collect the fermentation liquor. Then the residue was treated with 1N NaOH for one hour at 60°C. The residue was washed 3 times with distilled water and drained completely and weighed. Some amount of wet weight was used for carotenoids estimation (Sacchindra, *et al.*, 2007). The wet residue was dried at 60°C for one hour.

Ash, moisture and protein contents in the fresh waste without fermentation and after fermentation by using natural probiotic for 24, 48 and 72 hrs were measured. Moisture and ash contents were determined as per AOAC method (AOAC, 1995). Protein content was estimated by Lowry method after digesting 100 mg dried material with 10 ml of 0.5 N NaOH for 2hrs at 40°C (Lowry, *et al.*, 1951). Chitin was estimated as done by Spinelli *et al.*, (Spinelli, *et al.*, 1974). Demineralization and deproteinization were expressed as percentages as described by Rao *et al.*, (2000).

$$\%DP = \frac{[(P_O \times O) - (P_R \times R)]}{P_O \times O} \times 100$$

P_o and P_r are protein concentrations before and after fermentation. O and R represents (g) of original sample and fermented residue in dry wt.

$$\%DM = \frac{[(A_O \times O) - (A_R \times R)]}{A_O \times O} \times 100$$

Where A_o and A_r represents ash in original and fermented residue. The fermentation liquor and wet residue were analysed for their total carotenoid (Sacchindra, *et al.*, 2007). The sum of total carotenoids extracted in both fermentation liquor and residue was compared to the total carotenoid content in the original shrimp biowaste to express the recovery percentage.

RESULTS AND DISCUSSION

General Analysis

In the present investigation the shrimp waste used had a moisture content of 81.5%, the protein 29.3%, ash 18% and chitin 12.85% on dry weight basis. The shrimp biowaste had an alkaline pH (8.10± 0.10) of wet waste. The results of screening experiments and information on load of inoculum for gram of shrimp biowaste after addition of inoculum and during fermentation were presented in Table.1. From the Table 1 the natural probiotic was found to be the best culture for obtaining a low pH of 3.01± 0.01 after 72 hrs and was significantly different from all other Lactic acid bacterial cultures. Thus natural probiotic was chosen for standardization of fermentation conditions and further studies.

Table.1. Inoculum level in the fermentation mixture along with changes in pH of shrimp (*Penaeus monodon*) biowaste fermented with different lactic acid bacteria (LAB) along with natural probiotic.

Bacterial strains	Lab load ^a	pH		
		24 h	48 h	72 h
<i>Lactobacillus plantarum</i> (ATCC 8014)	5%	4.920±0.917	4.667±0.04	4.345±0.04
<i>Lactobacillus pentosus</i> (ATCC8041)	5%	4.932±0.940	4.569±0.08	4.335±0.03
<i>Lactobacillus Fermentum</i> (NRRL B-1840)	5%	4.934±0.927	4.606±0.09	4.352±0.04
Probiotic	5%	3.18±0.01	3.05±0.01	3.01±0.01

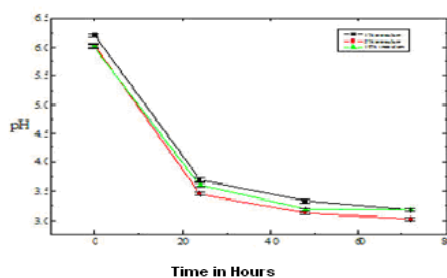
pH of the original shrimp biowaste was 8.10.

^aTotal bacterial strain load in the shrimp waste % inoculum

Effect of inoculum level

Shrimp biowaste was treated at the start of fermentation with 1, 5 and 10 (v/w) of natural probiotic in the presence of 15% (w/w) glucose for a period of 72hrs. The effect of inoculum level on pH reduction of shrimp biowaste was shown in Figure.1. The pH was decreased from 6.20 to 3.01 as the incubation period increases from 0 to 72 hrs for all inoculum levels (1, 5, 10%). However, the pH of 5% inoculum level was lower than the pH of 1% and 10% inoculum levels for all incubation period. Similar inoculum level (5%) in case of *Lactobacillus* species has been reported (Shirai, *et al.*, 2001). The maximum decrease of pH was obtained for 5% inoculum level due to production of lactic acid and acetic acid by microorganisms present in natural probiotic. Hence the inoculum level of 5% was chosen as optimum for further studies.

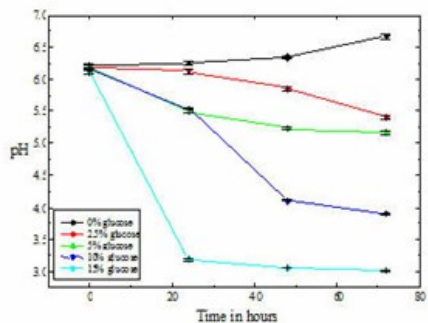
Figure.1. Effect of probiotic inoculum level (% v/w) on changes in pH of shrimp biowaste during fermentation with 15g/100 (% w/w) glucose concentration.



Optimization of glucose concentration for pH reduction

Shrimp biowaste is a poor source of fermentable carbon. Several carbohydrate sources have been studied to carry out a lactic acid ensilation of shrimp (Hall and Da Silva, 1992; Cira, *et al.*, 2002). Glucose, lactose, malt or whey powder must be added to medium for growth (Cira, *et al.*, 2002). Glucose was chosen in this study for being readily fermentable sugar. Shrimp biowaste was fermented with a fixed amount of inoculum (5% v/w) and variable concentrations of glucose 0, 2.5, 5, 10 and 15% w/w. The results obtained were shown in Figure.2. For the absence of glucose concentration (0%) the pH was increased from 6.21 to 6.67 as the incubation period increases from 0 to 72hrs. This was because of absence of substrate.

Figure.2. Effect of Glucose concentration (% w/w) on changes of pH during fermentation of Shrimp biowaste with 5% inoculum



For the four glucose concentrations of (2.5, 5,10 and 15%) the pH values decreased as the incubation period increases for 0 to 72 hrs and the pH values obtained at the end of incubation period 72 hrs were 5.4, 5.17, 3.9 and 3.01. This was due to fermentation of glucose to lactic acid by microorganisms present in natural probiotic. The initial quantity added has been reported to be a critical factor in lactic acid fermentation of shrimp wastes (Laura RC, *et al.*, 2006; Patidar P, *et al.*, 2005). Similar result was reported by many other researches [Mathew and Nair, 2006; Rao, *et al.*, 2000). Beyond 15% glucose concentration the pH was increased this may be due to product inhibition caused by excess amount of substrate (N. Bhaskar, *et al.*, 2007). Hence, the 15% glucose concentration was chosen as optimum for further studies.

Effect of incubation time

The effect of incubation period was shown in Figure.3. The pH was decreased from 6.20 to 3.01 and TTA was increased from 0.99 to 2.713 as the incubation period increases from 0 to 72hrs. The significant reduction in pH as a result of acids produced by lactic acid culture in the natural probiotic. Thus the optimized incubation time obtained 72hrs was chosen for lowest pH of 3.01 and increased in TTA of 2.713.

Figure.3. Effect of pH and TTA on shrimp biowaste during fermentation with 5% inoculum and 15g / 100(w/w) glucose concentration.

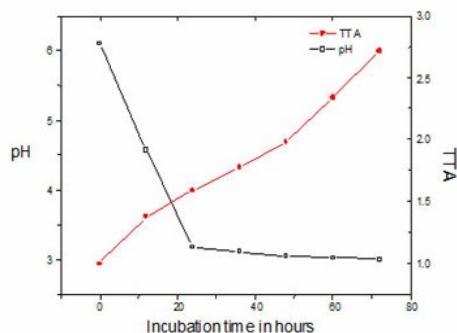
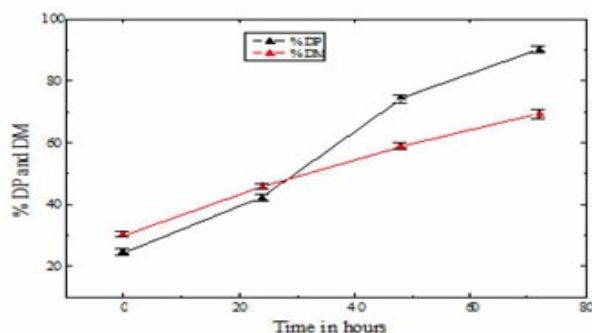


Figure.4. Effect of Demineralization and Deproteinization by 5% probiotic with 15g/100 (w/w) glucose concentration of shrimp biowaste during fermentation



Effect of fermentation on recovery of chitin and carotenoids

Under optimal conditions the effect of fermentation on demineralization (DM) and deproteinization (DP) of shrimp biowaste were presented in Figure.4. The best demineralization efficiency of 69% and deproteinization of 89% were obtained at 15% glucose concentration and 5% inoculum and incubation period of 72hrs. Fermentation by using probiotic which contains lactic acid bacteria is an effective technique for decomposition of shrimp biowaste results in silage comprising of protein rich liquor and insoluble chitin. During fermentation, due to deproteinization and demineralization the protein and ash content in the residue decreases and liquor portion becomes rich in proteins. Protein present in shrimp biowaste is hydrolysed due to action of endogenous proteolytic enzymes and proteases produced by lactic acid bacteria present in probiotic resulting in deproteinization. This clearly indicates the usefulness of fermentation as a tool to produce chitin biologically instead of conventional chemical methods that use strong mineral acids and strong alkalis.

Proteolysis results in better recovery of carotenoids from shrimp biowaste. The recovery of carotenoids during fermentation was presented in Table 2. The table shows the recovery of carotenoids was more than 72% at the end of 72hrs. It can be observed that carotenoid content in residue was lower than in the filtrate. This could be due to the fact that residue undergoes washing before estimating carotenoid content. In addition the presence of protein rich liquor portion is indicative of its uses as human or animal food (Rao, *et al.*, 2000).

Table.2. Effect of Probiotic on the recovery of carotenoid content (μg) of shrimp biowaste (original wet weight of biowaste – 100g)

Sample	Residue wet weight (g)	carotenoid (μg) X	Filtrate volume (ml)	carotenoid (μg) Y	Total carotenoid content (μg) (X+Y)	carotenoid(% of original)
0 h	34.4 \pm 1.0 ^a	2894 \pm 150	104 \pm 2	1561 \pm 45	4455 \pm 190 ^a	77.6 \pm 1.8
24 h	35.2 \pm 1.2 ^b	1538 \pm 50	100 \pm 3	2745 \pm 25	4283 \pm 75 ^b	74.6 \pm 1.0
48 h	26.2 \pm 0.8 ^c	1783 \pm 45	135 \pm 3	2355 \pm 40	4138 \pm 85 ^c	72.0 \pm 1.2
72 h	26.7 \pm 0.6 ^c	1789 \pm 50	140 \pm 4	2380 \pm 110	4169 \pm 160 ^c	72.6 \pm 2.1

Fresh 5740 \pm 55 μg in 100g wet waste. Values with similar letters in the same column are not significantly different ($P \geq 0.05$). Carotenoid content in original taken as 100%.

Conclusions

The present study shows that the probiotic was efficient inoculum in fermentation of the shrimp biowaste. The bioremediation performances are strongly affected by parameters such as inoculum levels, glucose concentrations, pH, TTA, DP and DM percentages. Fermentation also allowed recovery of carotenoids. The protein rich fermentation liquor containing carotenoids could be effectively utilized by concentrating it with other feed ingredients. Further studies on physico-chemical characteristics of chitin prepared using biological methods needed to ascertain its superiority over chitin prepared by chemical methods.

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