IN VITRO EVALUATION OF ANTIBACTERIAL ACTIVITY OF CHITOSAN EXTRACTED FROM MULBERRY SILKWORM (BOMBYX MORI) PUPAE

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ABSTRACT

The mulberry silkworm pupae, one of the major by-products of silk industry is considered as waste in silk reeling industries. Disposal of silkworm pupae after reeling silk is a big concern causing health hazards and pollution to the environment. Mulberry silkworm pupae contain numerous biological constituents which are of great value as feed for animals, human beings, medicine and manure for crops. These constituents of pupae are being used in diverse sectors for various purposes. Pupal skin is made up of chitin and chitosan, which is a polysaccharide (N acetyl glucosamine) and is the second most abundant material available on earth. To elucidate more information on pharmaceutical utility of silkworm pupae, the present study focused on extraction of chitosan (deacetylated chitin) from silkworm pupae and antibacterial activity was determined. The deacetylated chitin was evaluated in vitro for antibacterial activity against gram negative (Escherichia coli) and gram positive bacteria (Bacillus thuringiensis, Staphylococcus aureus, and Enterococcus faecalis). Different concentrations of chitosan such as 10, 30, 50, 100, 250, 500 and 750 µl were used for this study. Among the different concentrations 750 µl /ml showed 17.5 mm, 15.0 mm, 11.5 mm and 14.0 mm of inhibition against E.faecalis followed by E.coli, S. aureus and B. thuringiensis. The zone of inhibition was increased with increasing concentrations of chitosan. The antimicrobial activity of chitosan indicated that the pupae generated from silk reeling industries could be used as an effective antimicrobial agent in the pharmaceutical industry.

KEYWORDS: Silkworm Pupae, by Product, Chitosan, Gram Positive Bacteria, Gram Negative Bacteria, Antibacterial Activity.

INTRODUCTION

Pupa, which accounts the major portion of the cocoon weight, is an inevitable by-product generated in large quantities (75-85%) in silk reeling industries and grainages which are discarded, as such, after reeling of silk thread causing pollution problems. It is estimated that 1.5 lakhs tonnes of pupae are produced annually in case of mulberry silkworm in India.
Attempts were made to utilize by-products of sericulture conventionally like silkworm excreta, rearing bed waste, molted skin, host plant shoots, sericin, silkworm pupae, silkworm moths as protein supplement for poultry, carps, fish, rabbits, piggery and dogs and as a compost (Rangacharyulu, 2003 and Dandin et al., 2005). Dry silkworm pupae contains 45-49% of protein and 23-24% of oil (Jolly et al., 1974), chitin (3.73 %) and chitosan (2.2 %) thus forming an important biosource of oil and proteins (Singh and Suryanarayana, 2003). And after extraction of these proteins and oil, the remaining waste is used for the extraction of chitosan, which is also another important by-product from sericulture industry (Katti et al., 1996).

Chitosan is a deacetylated form of chitin is the most abundant among the natural polysaccharides. It forms the basis of main constituent of the outer skeleton of insects (Vishu Kumar et al., 2005). It has gained the attraction of the scientific community due to its antimicrobial activity, biodegradability, wound healing activity, antitumour activity and immune enhancing effect. Chitosan possesses higher antibacterial activity and lower toxicity towards mammalian cells.

Most of the sericultural farmers belong to economically weaker sections and unaware about the potential applications of by-products in diverse fields. The value addition through utilization of by-products was never thought of. However, the conventional use of the by-products will not provide higher monetary returns to the poor rural farmers. In order to make the sericultural industry more profitable, the waste products should be converted into biologically active substances with important uses in pharmaceutical, cosmetic, paper and cellulose, and organic agricultural food industries.

The idea of by-product utility can be highly useful to sericulture industry and help in not only increasing the socio economic status of the rearers but also effective conversion of wastes / by-products into highly useful biological products which in turn will proportionately reduce the silk production cost and environmental problems. Hence, the present study was undertaken on the conversion of silkworm pupal waste into eco friendly biological products i.e., chitosan and evaluation of its antibacterial activity.

Materials and Methods

Extraction of chitin and chitosan from silkworm pupae

The chitin and chitosan extraction involved mainly three steps viz., Deproteinization, Demineralization and Deacetylation. Deproteinization of silkworm pupae was carried out by
using 4 per cent dilute sodium hydroxide at 70°C for 4 hours. Silkworm pupae to NaOH ratio of 1:10 (w/v) were maintained. After the treatment, the materials were washed with running tap water for 4-5 times to remove excess alkali and subsequently rinsed in deionized water. Demineralization of silkworm pupae was carried out by treating 3 per cent Hydrochloric acid at ambient temperature for two hours with deproteinized pupae to liquid ratio of 1:10 (w/v). The material was washed with running water and rinsed in deionized water. The product obtained was chitin. Chitin was dried in hot air oven for 12h at 50°C for further use.

Deacetylation was carried out by treating chitin with 45 per cent concentration of sodium hydroxide at 95°C for 4 hours and the solid to liquid ratio was maintained at 1:12 (w/v). After the treatment, the material was washed with water and rinsed in deionized water. The final product obtained was chitosan. The chitosan was dried in hot air oven for 10 h at 50°C for further use (No and Meyers, 1995).

**In vitro evaluation of antibacterial activity of chitosan against bacterial pathogens**

The antibacterial activity of chitosan was evaluated *in vitro* against silkworm pathogens viz., *Bacillus thuringiensis*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*. The chitosan of different concentrations such as 10, 30, 50, 100, 250, 500 and 750 µl were used and experiment was carried out by using disc diffusion technique. Nutrient agar was prepared and sterilized using autoclave at 121°C for 15 minutes. It was then poured on to petri plates and allowed to solidify. Pure culture of silkworm pathogens was spread on to agar plates so as to achieve a confluent growth. The discs was prepared and impregnated with different concentrations of chitosan. Streptomycin was used as control. The impregnated disc was placed on the surface of the medium at three points equidistant from one another. Then the plates were incubated at 37°C for 24 hours. The diameter of inhibition zone was measured after 24 hours incubation.

**Results and Discussion**

The antibacterial activity of chitosan was evaluated *in vitro* against *E.coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus thuringiensis* based on clear inhibition zone. The zone of inhibition was measured and given in the table 1. It is assumed that there is no antimicrobial activity, if there is no inhibition zone in the chitosan spotted place. The average size of inhibition zones varied from 4.5 – 15.0 mm against *E.coli*, 4.5-
11.5 mm against *S.aureus*, 8.5 – 17.5 mm against *E. faecalis* and 4.0 – 14.0 mm against *B. thurigiensis*. Chitosan markedly inhibited the growth of organisms. Among the different concentrations 750 µl /ml showed 17.5 mm, 15.0 mm, 11.5 mm and 14.0 mm of inhibition against *E. faecalis* followed by *E.coli*, *S. aureus* and *B. thuringiensis*. The control (streptomycin) showed the inhibition zone of 9.0, 12.0, 14.0 and 7.0mm against *E.coli*, *S. aureus*, *E. faecalis* and *B. thuringiensis*. The results indicated that the zone of inhibition was increased with increasing concentrations of chitosan. Among the organisms, *E. faecalis* is more susceptible to chitosan followed by *E.coli, B.thuringiensis* and *S.aureus*. It was found that there was no antibacterial activity at 10µl for all the four bacterial organisms and 30µl for *E. faecalis* and *B. thuringiensis*. As acetic acid was used to dissolve the chitosan, it was also taken for antibacterial activity study and it showed no antibacterial activity against any of the tested organisms.

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<tr>
<th>Sl.No.</th>
<th>Concentration of chitosan µl /ml</th>
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<td><em>E.coli</em></td>
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<td>1.</td>
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<td>5.</td>
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<td>6.</td>
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<td>7.</td>
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<td>8.</td>
<td>Control (streptomycin)</td>
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**Table 1. Antibacterial activity of chitosan against silkworm bacterial pathogens**

The antibacterial activity might be the interaction between positively charged chitosan and negatively charged microbial membranes, which prevents the transport of essential solutes into the cell and results in leakage of proteinaceous and intracellular components thereby killing the bacterial cell (Chung *et al.*, 2011). The antibacterial activity of chitosan was studied and reported by several workers. The present finding was supported by the reports of Salmabi and Seema (2013) who studied the antibacterial activity of chitosan against *E. faecalis* (17.0 mm) and *S. aureus* (14 mm) at 400µg. The present study showed the antibacterial activity of 17.5 mm and 11.5 mm against *E. faecalis* and *S. aureus*. The antibacterial activity of *S. aureus* was proved by Islam *et al* (2011) and supported the present findings. In gram positive bacteria, the cell membrane is covered by a cell wall consisting of layers of peptidoglycans which contains acetylmuramic acid as well as D- and L- amino acids.
and techoic acid (Tortora, 2010) to which the positively charged amino groups of chitosan binds, result in cell wall distortion – disruption and expose cell membrane to osmotic shock and exudation of cytoplasmic contents (Vishu kumar et al., 2005). The binding of chitosan to techoic acids coupled with a potential extraction of membrane lipids results in bacterial death. The reduced inhibition activity of *S. aureus* might be the increased molecular weight of chitosan (Liu et al., 2006).

No et al (2002) reported that the antibacterial activity of chitosan is effective in inhibiting growth of bacteria. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium. Chitosan generally showed stronger bactericidal effects for positive bacteria than negative bacteria. The antimicrobial activity is associated with molecular weight, degree of acetylation, concentration of chitosan and load of pathogen (Fernandes et al., 2008). Liu et al. (2004) showed that chitosan acetate solution increased the permeability of outer and inner membranes of gram negative bacteria *E.coli*, and this damage was caused by electrostatic force of interaction of NH\(^{+}\) groups of chitosan acetate and phosphoryl groups of phospholipids of cell membrane.

Tareq et al (2013) reported that lower molecular weight chitosan is more effective against gram negative bacteria whereas, high molecular weight is effective against gram positive bacteria. The study showed the activity of both gram negative and gram positive bacteria. These pathogens are responsible for causing disease in silkworm.

The antibacterial activity of gram positive or gram negative is however, somewhat controversial. Some authors stated that chitosan generally showed stronger effects for gram positive bacteria (No et al., 2002). Dutta (2009) reported that hydrophilicity in gram negative bacteria is higher that in gram positive bacteria make more sensitive to chitosan. Masson (2008) indicated that the charge density on the cell surface is a determinant factor to establish the amount of adsorbed chitosan. This would suggest that the antibacterial mode of action is dependent on host microorganisms.

The present study demonstrated the chitosan extracted from silkworm pupae had more antibacterial activity which inhibits both gram negative and gram positive bacteria. Effective utilization of silkworm pupae by value addition finds application as a antimicrobial agent in pharmaceutical industry and thereby, conversion of waste into biological product.
Acknowledgement

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References