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Antimicrobial potential of chitosan extracted from *Bacillus* sp. by optimization of growth culture

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Chitosan (β-1,4-D-glucosamine), the deacetylated form of chitin, owing to its unique biocompatible, biodegradable, nonantigenic and nontoxic potential, has multiple applications, such as nanoparticles synthesis, drug delivery, dye removal, and as thickening as well as antimicrobial agent. However, production of chitosan involves harsh chemical process. It is neither economical nor environment friendly. Apart from marine organisms, bacteria are also good source of chitosan. Here, we report an alternative cost effective method for production of chitosan from enzymatic deacetylation of bacterial chitin under controlled laboratory conditions. We screened bacterial strains from East Kolkata Wetland area that acts as a natural incubator for microorganisms with rich diversity and identified potential chitosan producing strains. The bacterial isolates, BRS 5 and PS 4 yielded 2 and 0.3% chitosan, respectively. The positive colonies (BRS 5 and PS 4) also showed antibacterial and antifungal activity against *E. coli* and *Candida albicans* ATCC 60193. The production of chitosan was optimized by optimizing the bacterial growth against different carbon source, such as glucose, lactose, maltose, fructose and starch in different pH (4-9), and different temperatures (20-45°C) to achieve an increased production rate.

Keywords: Antibacterial, Antifungal, Chitin deacetylase activity, Enzymatic deacetylation

Bacterial infectious diseases emerge day-by-day with new virulent forms and new epidemiological settings necessitates development of new compounds with antimicrobial activities preferably from natural economical sources[1-4]. In recent years, chitosan (β-1,4-D-glucosamine), has gained a lot of research interest due to its unique properties like biocompatibility, biodegradability, non toxicity and nonantigenicity[5], oil sorption/retention[6], defluoridation[7], enzyme production[8], etc. It has wide applications in wound healing, drug carrier, drug delivery[9,10] system, food & beverage[11,12] apart from its role as thickening agent in fabric printing[13], in manufacturing fibre board[14], biomedical[12], pharmaceutical[12] and paper industry[15], waste water treatment[12] and cleaning environmental contamination, particularly by heavy metals[16,17], dye removal[18,19], plant productivity[20], etc. It also posses antiviral, antimicrobial[3,4], antioxidant and antitumour activities[21]. Chitosan is present in many marine invertebrates, shells of fish, shrimp[3,16,22], crab[9,14], insects, cells of fungi, bacteria and yeasts in the form of chitin and occurs in white, hard crystalline form of nitrogenous polysaccharide. Among the different sources mentioned, prawn is one of the most widely discussed[12,22]. Different sources of prawns and shrimps, such as *Penaeus monodon*, *P. indica P. merguiensis*, *Litopenaeus vannamei* and *Fenneropenaeus indicus*, *F. semisulcatus* are known to be used for chitosan extraction[23-25]. Micro-organisms like fungi and bacteria can also release chitosan[8]. Various strains of *Aspergillus niger* and *Salmonella typhi*, *Salmonella paratyphi* A, *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa*, *Bacillus* sp. and *Serratia* sp. were also previously obtained for chitosan production[26].

Chitin is converted to chitosan either by enzymatic preparations or by chemical hydrolysis. However, chitin is not soluble in water or in the majority of organic solvents but chitosan, prepared from chitin through chemical N-deacetylation, is water soluble and possesses biological properties as mentioned above[15]. Global market size for chitosan has been estimated at 6.8 billion USD for the year 2019 which is expected register a compound annual growth rate of 24.7% during 2020 to 2027[12]. In this context, here, we explored production of chitosan by eco-friendly bacterial strains-mediated enzymatic deacetylation of chitin, and also studied the antimicrobial effect of the bacterial strains.

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Materials and Methods

Collection and preparation of sample

Soil sample was collected from the root of Brahmi plant and also from different other agricultural fields of East Kolkata Wetland and stored in sterile containers. The collected samples were air dried for few hours and then oven dried at 25°C. After drying, the soil was passed through a stainless steel sieve to remove other impurities and used for further analysis.

Isolation of bacteria

Bacterial strains were isolated from the soil using initial screening in normal saline (0.9%) by dilution plating method on nutrient agar plate. Plates were incubated at 37°C for 24 h. Colonies were re-streaked on nutrient agar for single colony isolation. Pure cultures were preserved as glycerol stock and stored at −20°C.

Biochemical characterization

Before moving for further screening processes the colonies were checked under microscope for Characteristics like colony morphology (colour, shape, Gram reaction). The single colonies were also analyzed for their biochemical characterization by Bergey's manual of systematic bacteriology[27].

Isolation of chitosan producing bacteria

All single bacterial colonies were screened on selective medium (chitin 1%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, P and N 0.05%, and agar 2%) and incubated for 2 days at 30°C. Bacteria having chitin degrading activity observed with their growth[28].

Screening for chitin deacetylase activity (CDA)

The screening of CDA involves preparation of p-nitroacetanilide solution from acetanilide. Then in a mixture of p-nitroacetanilide and ethanol, filter paper strips were cut and immersed and air dried. This was repeated for three times to ensure the strips having sufficient concentration of p-nitroacetanilide. Then the strips were dipped in a 5 mL of 24 h old culture fermentation medium (1.0 g of yeast extract, 0.4 g of ammonium sulfate, and 0.15 g of potassium dihydrogen phosphate for 1000 mL) containing previously isolated colonies and incubated at 25ºC for 24 h. After incubation, the development of yellow colour in the strip indicates the presence of deacetylase in respective bacterial colonies[28].

Extraction of chitosan from bacterial chitin

In a flask containing 50 mL of fermentation medium, 1.0 mL of positive bacterial suspension was inoculated. One flask was not inoculated with any organism and used as control. All the flasks were incubated on rotary shaker at 25ºC for two days. After incubation, each flask was used for chitosan recovery[28].

Recovery of chitosan from bacterial species

As the fermented broth not only contains chitosan but also other impurities it needs several centrifugation steps to collect the pure form. At first, the flasks were centrifuged at 5000 rpm, the supernatant was discarded, and then the pellet contained mixture of bacteria, chitin and chitosan. About 10 mL of 0.1N NaOH was added to the pellet, mixed thoroughly and autoclaved. Most of the cells were solubilized during this alkaline treatment. The tubes were again centrifuged at 12000 rpm and then the pellets contain chitin, chitosan, and small amount of cell debris. This was mixed with 10mL of 2% acetic acid and mixture was left on a shaker overnight at room temperature (25°C) to solubilize chitosan. The contents were again centrifuged at 12000 rpm. Pellet was discarded and 10 mL supernatant was collected and the presence of chitosan in it was checked by the formation of white precipitate upon neutralization with 1N NaOH[28].

Qualitative and quantitative estimation of chitosan

The white precipitate was centrifuged at 5000 rpm. It was washed twice with distilled water (pH 7). Then precipitate was re-suspended in 0.5 mL of distilled water (pH 7) and this suspension was taken in watch glass. It was allowed to oven dry. On the dried precipitate, 2-3 drops of iodine solution was added to get brown coloured precipitate. The mixture was acidified with 2-3 drops of 1% H_2SO_4 where the brown colour should change to purple. This indicates the presence of chitosan and also that the microorganism is a chitosan producing bacteria. For the estimation of quantity, weight of clean and dried Petri plates were taken. Then the precipitate of chitosan was re-suspended in 1mL of distilled water and poured in Petri plates. It was kept at oven drier. After drying, plates were again weighed to get the produced amount of chitosan[28].

Optimization of bacterial growth using different carbon source, pH and temperature

The effect of carbon source was determined with glucose, fructose, lactose, maltose and starch. Each source was used at a concentration ranging from 0.1-1%. The bacterial growth was determined after 24 h of incubation at 37°C using UV-Vis spectrophotometer at

640 nm. The positive isolates were checked for their growth in different pH values (4-9) adjusted with acidic and basic solutions. All the test tubes were incubated for 24 h at 37°C and the growth was observed in UV-Vis spectrophotometer at 640 nm. The colonies were also checked for the optimum growth at different temperature (20-45°C). Each of the culture was incubated at different temperatures for 24 h. The growth was measured using UV-Vis spectrophotometer at 640 nm[29].

Antimicrobial activity of chitosan producing bacteria

The antimicrobial activity of the positive bacterial isolate was checked against *E. coli* and *Candida albicans* ATCC 60193 by disc diffusion method[30].

Statistical analysis

Standard deviation and standard error was performed against the zone of inhibition obtained for the two bacterial strains using Microsoft office excel.

Results

Isolation and characterization of chitosan producing bacteria

The bacterial colonies were isolated from soil samples in two different time intervals of a year. Surprisingly they exhibited same morphological characteristics (Table 1). on the basis of their colour, colony morphology (shape) and cell morphology (Gram reaction) according to Bergey's Manual of Systematic Bacteriology All the bacterial cultures were gram positive, endospore forming, rod-shaped bacterium with catalase positive, citrate positive, glucose-fructose-starch positive, mannose-lactose negative, MR-negative, and VP negative (Table 2). They were determined by the growth on selective medium. The results identified the isolates as *Bacillus* sp.

Screening of cultures for chitin deacetylase activity

As the enzyme chitin deacetylase is responsible for the production of chitosan in bacteria it can be assumed that the positive isolates are potent chitin degraders and they would also produce the enzyme chitin deacetylase so as to release chitosan. Therefore, the positive isolates which were screened for chitin deacetylase activity (Table 3) using the diagnostic strip changed the colour to yellow because of the enzyme activity.

Confirmation of chitosan production

The fermented broth after incubation was tested for the presence of chitosan. Only two isolates i.e., BRS 5 and PS 4 gave positive results. The precipitate

Table 1 — Morphological characters of isolates

Isolate	Shape	Size	Colour	Organism
BRS1	Rod	Small	Purple	*Bacillus* sp.
BRS2	Rod	Small	Purple	*Bacillus* sp.
BRS3	Rod	Small	Purple	*Bacillus* sp.
BRS4	Rod	Small	Purple	*Bacillus* sp.
BRS5	Rod	Small	Purple	*Bacillus* sp.
BRS6	Rod	Small	Purple	*Bacillus* sp.
BRS7	Rod	Small	Purple	*Bacillus* sp.
BRS8	Rod	Small	Purple	*Bacillus* sp.
BRS9	Rod	Small	Purple	*Bacillus* sp.
BRS10	Rod	Small	Purple	*Bacillus* sp.
BRS11	Rod	Small	Purple	*Bacillus* sp.
BRS12	Rod	Small	Purple	*Bacillus* sp.
BRS13	Rod	Small	Purple	*Bacillus* sp.
BRS14	Rod	Small	Purple	*Bacillus* sp.
BRS15	Rod	Small	Purple	*Bacillus* sp.
BRS16	Rod	Small	Purple	*Bacillus* sp.
BRS17	Rod	Small	Purple	*Bacillus* sp.
BRS18	Rod	Small	Purple	*Bacillus* sp.
BRS19	Rod	Small	Purple	*Bacillus* sp.
BRS20	Rod	Small	Purple	*Bacillus* sp.
PS1	Rod	Small	Pink purple	*E. coli*
PS2	Rod	Small	Pink	*E. coli*
PS3	Rod	Small	Pink	*E. coli*
PS4	Rod	Small	Purple	*Bacillus*sp
PS5	Rod	Small	Purple	*Bacillus*sp
PS6	Rod	Small	Purple	*Bacillus*sp
PS7	Rod	Small	Purple	*Bacillus*sp
PS8	Rod	Small	Pink	*E. coli*
PS9	Rod	Small	Pink	*E. coli*
PS10	Rod	Small	Purple	*Bacillus*sp
PS11	Rod	Small	Purple	*Bacillus*sp
PS12	Rod	Small	Purple	*Bacillus*sp
PS13	Rod	Small	Purple	*Bacillus*sp
PS14	Rod	Small	Pink	*E. coli*
PS15	Rod	Small	Pink	*E. coli*
PS16	Rod	Small	Pink	*E. coli*

Table 2 — Biochemical characterization of isolated strain

Basic characteristics	Properties
Catalase	Positive (+ve)
Citrate	Positive (+ve)
Gram staining	Positive (+ve)
Indole test	Negative (+ve)
Motility test	Positive (+ve)
Methyl red test	Negative (-ve)
Oxidase	Negative (-ve)
Shape	Rod
Spore	Positive (+ve)
VP test	Negative (-ve)
Arabinose	Negative (-ve)
Fructose	Positive (+ve)
Glucose	Positive (+ve)
Starch	Positive (+ve)
Mannose	Negative (-ve)
Lactose	Negative (-ve)
Manitol	Negative (-ve)
Acetate utilization	Positive (+ve)
Lysine	Negative (-ve)
Phenyl alanine Deaminase	Negative (-ve)

Table 3 — Display of chitin deacetylase activity by isolates

Organism inoculated	Initial colour	Colour after incubation (24 h)	Chitin deacetylase activity
BRS1	Colourless	Colourless	-
BRS2	Colourless	Yellow	+
BRS3	Colourless	Colourless	-
BRS4	Colourless	Colourless	-
BRS5	Colourless	Yellow	+
BRS6	Colourless	Colourless	-
BRS7	Colourless	Colourless	-
BRS8	Colourless	Colourless	-
BRS9	Colourless	Colourless	-
BRS10	Colourless	Colourless	-
BRS11	Colourless	Colourless	-
BRS12	Colourless	Colourless	-
BRS13	Colourless	Yellow	+
BRS14	Colourless	Colourless	-
BRS15	Colourless	Colourless	-
BRS16	Colourless	Colourless	-
BRS17	Colourless	Colourless	-
BRS18	Colourless	Colourless	-
BRS19	Colourless	Colourless	-
BRS20	Colourless	Yellow	+
PS1	Colourless	Colourless	-
PS2	Colourless	Colourless	-
PS3	Colourless	Yellow	+
PS4	Colourless	Yellow	+
PS5	Colourless	Colourless	-
PS6	Colourless	Yellow	+
PS7	Colourless	Colourless	-
PS8	Colourless	Colourless	-
PS9	Colourless	Colourless	-
PS10	Colourless	Colourless	-
PS11	Colourless	Colourless	-
PS12	Colourless	Colourless	-
PS13	Colourless	Colourless	-
PS14	Colourless	Colourless	-
PS15	Colourless	Colourless	-
PS16	Colourless	Colourless	-
Control	Colourless	Colourless	-

obtained have confirmed the presence of chitosan by the display of dark purple colouration. The bacterial strain BRS 5 yielded 2% (per 100 mL) whereas PS 4 yielded only 0.3.

Optimization of bacterial strains with carbon, pH and temperature

Any bacterial species has a profound influence of medium composition, carbon source, pH, temperature on its growth, activities, survival etc. Here for the bacterial strain BRS 5, the growth is maximum in starch at a concentration of 0.8% followed by glucose and lactose (Fig. 1A) whereas a minimum growth was observed in fructose. On the other hand strain PS 4 showed high growth in Lactose at a concentration of 0.7% followed by starch (Fig. 1B). Specific pH range for the growth of any bacteria is between 4 and 9 and the optimum growth usually occur between 6.5 to 7.5. But in this study optimum growth for BRS 5 and PS 4 was observed at pH 8.5 and 7, respectively (Fig. 1C). Temperature also plays an important role in the growth of microorganisms. The strain BRS 5 showed optimum growth at a temperature of 40°Cand PS 4 at 35°C (Fig. 1D).

Antimicrobial activity of chitosan producing strains

The antimicrobial activity was performed against a gram negative bacteria *E. coli* and a fungal strain *Candida albicans* ATCC 60193 and both the strains, BRS5 (Fig. 2 A and D) and PS4 (Fig. 2 B and C) showed antimicrobial activity The results are given in the following standard deviation graph (Fig. 3) which clearly shows PS 4 having better antimicrobial activity than BRS 5.

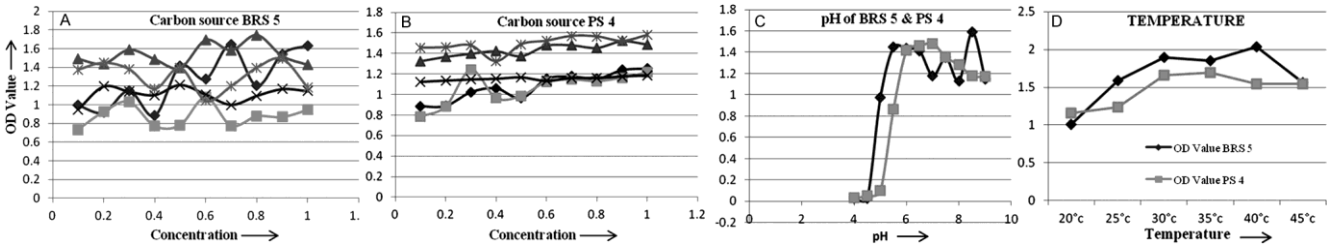

Fig. 1 — Optimization using different source (A) Carbon source for BRS 5; (B) Carbon source for PS 4; (C) pH value; and (D) Temperature

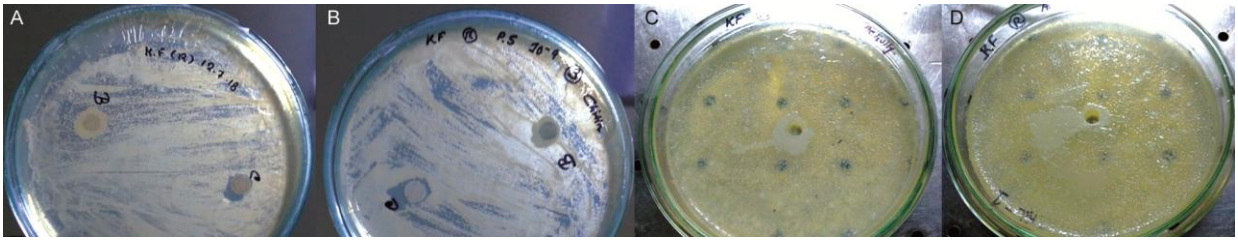

Fig. 2 — Zone of inhibition: (A) BRS 5 against *E. coli*, where B-BRS 5 and C-control; (B) PS 4 against *E. coli*, where B-BRS 5 and C-control; (C) PS 4 against *C. albicans*; and (D) BRS 5 against *C. albicans*

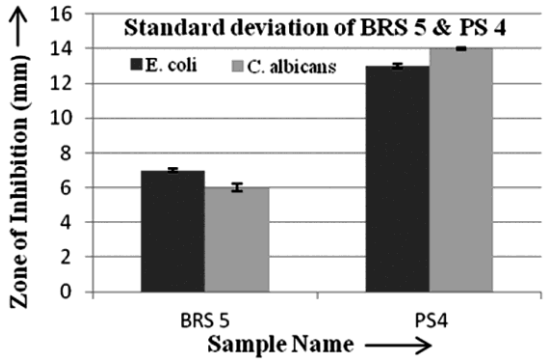

Fig. 3 — Standard deviation of BRS 5 and PS 4 against antimicrobial activity

Discussion

As chitosan has a wide range of uses in many fields such as biological, pharmaceutical, agricultural, food industry, water industry, etc. due to its biocompatibility and nontoxic characters so the need of its production also increasing day-by-day. Chitosan can be produced from chemical treatments but the quality may decreases with respect to its properties like 'Degree of Deacetylation (DD)', molecular weight, viscosity, etc. which tends to be the main factors of chitosan activity, particularly antimicrobial[31,32]. The acid soluble chitosan with 99% DD and lower viscosity effectively inhibited bacteria growth[32]. Alternatively, enzymatic treatment can help to minimize these drawbacks and use of bacterial species to produce chitosan offers the possibility of the development of a potentially good alternative process[33].

Zygomycetous and few edible basidiomycetous fungi have also gained attention for their chitosan producing ability with potential advantages in terms of homogenous polymer length, high degree of deacetylation and solubility[34]. Bacterial strains with chitin deacetylation (CDA) capacity *viz*., *Bacillus licheniformis*[35], *B. subtilis*[36], *B. thermoleovorans*[37], *Rhodococcus equi*[38], etc. are reported rare. Hence, there is a need to search for suitable bacterial strains having CDA producing ability to increase the existing list. In this work, the two bacterial strains (BRS 5 & PS 4) which were isolated from the root soil of a Brahmi plant have shown positive results. Further, we identified the strains using different morphological and biochemical characterizations. The percentage of yield for BRS 5 and PS 4 was found to be 2.0 and 0.3%, respectively. This yield could be increased by optimizing the fermentation process with starch and lactose as carbon source and keeping the pH between 7 to 8.5. Accordingly, the CDA production rate could also be increased and thereby increasing the chitosan production rate. Chitosan have an effect on the type of bacteria living in the intestines or on the mode of action of these bacteria and can help prevent diseases such as colon cancer[39]. The antimicrobial activity is one of the most important and essential activities of any compound or polymer to be considered as functional in any biomedical industry[40,41].

Numerous researches have been carried out to understand the antimicrobial potential of chitosan[31,38-41]. Chitosan based novel biodegradable nanoparticles with primary amine groups in repeating units demonstrated better antibacterial and antitumor activity compared to chitin and chitosan[41]. The amine groups which is responsible for the positively charged condition of chitosan and interact with the negatively charged groups of bacterial cells and thereby inhibit the bacterial growth, particularly the Gram-negative bacteria rather than the Gram-positive bacteria. However, few others have shown better inhibition of Gram-positive bacteria. In this study, both the strains (BRS 5 & PS 4) have shown antimicrobial activity against the Gram-negative *E. coli* as well as Gram-positive fungal strain *C. albicans* similar to earlier reports where disaccharide chitosan derivatives have been shown to inhibit the Gram-positive *Staphylococcus aureus* as well as Gram-negative *E. coli*[32]. Molecular weight is also another important property for characterizing chitosan. Low molecular weight of chitosan can also act as a good antimicrobial agent as it can easily penetrate into the bacterial cell and inhibit the protein synthesis and ultimately inhibiting the bacterial growth. Zheng & Zhu[43] have shown increased inhibition proportional to the concentration for chitosan with molecular weight <300 kDa for Gram +ve *Staphylococcus aureus* but not for Gram –ve *E. coli*. Apart from the previously discussed properties pH, temperature and many other factors like bacterial strains and other biological conditions may affect the inhibition of bacteria. Kim *et.al*.[44] have indicated that the presence of phytochemicals such as caffeic acid, ferulic acid and sinapic acid in chitosan improves its antimicrobial activity.

Conclusion

Chitosan, as one of the richest polysaccharides and versatile in nature, has wide biomedical, environmental and pharmaceutical applications

including the antimicrobial activity. In this particular study, chitosan extraction was done by enzymatic method using bacterial strain. Increased concentration of the commercial media along with optimized environmental condition produced better yield revealing its potential for large scale economical and eco-friendly production of chitosan with potent antimicrobial activity from these bacterial strains.

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