International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2020; 8(1): 1408-1410

Received: 14-11-2019
Accepted: 18-12-2019

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Isolation and screening of microorganisms for chitosan production

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DOI: https://doi.org/10.22271/chemi.2020.v8.i1t.8455

Abstract
Chitosan is a cationic linear biopolymer that is produced by deacetylation of chitin by chitin deacetylase. This enzyme hydrolyses acetamido groups of N-acetyl glucosamine in chitin. Chitosan has immense potential especially in the field of agriculture, food and pharmaceuticals. In most of the countries, commercial exploitation of chitosan is predominantly done by conversion of chitin to chitosan by treating shellfish waste with alkali. The biological methods for extraction of chitosan were used as alternative for chemical methods. In the present study, 18 morphologically different fungal isolates, 17 bacteria and 3 actinomycete were isolated from soil and water samples. Out of these 38 strains, six isolates of fungi, two bacterial isolates and one actinomycete showed positive results by production of yellow color in the chitin agar media supplemented with p-nitroacetanilide as indicator.

Keywords: Chitosan, microorganisms, chitin deacetylase, fungi

Introduction
Chitosan is a homopolymer consisting of β (1-4) bonds between 2-amino-2-deoxy-D glucopyranose and 2-acetamido-2- deoxy-D-glucopyranose. It is derived from the deacetylation of chitin (β(1-4)-N-acetyl-D-glucosamine) which is the second most abundant polysaccharide found in nature after cellulose. Crustacean chitin is naturally closely associated with proteins, minerals, lipids and pigments. Chitin is insoluble in most common solvents while chitosan is soluble in diluted acid solutions. Chitosan is non-toxic, biocompatible, biodegradable, antimicrobial agent and has high charge density. It has been reported to have numerous applications especially in the field of agriculture, food and pharmaceuticals. The conventional method for the production of chitosan includes several steps such as demineralization with hydrochloric acid, deproteinization, deacetylation of crustacean chitin using hot concentrated caustic soda solution for a prolonged time, extraction of chitosan in acidic medium and again precipitation in alkaline medium. The crustacean chitosan is inconsistent in its physiochemical characteristics as a result of variability in raw materials, harshness of the isolation and conversion processes, and caustic effects of the chemicals used in the isolation process, variability in the levels of deacetylation and protein contamination (Abdou *et al.* 2008) [1]. This method of chitosan production has adverse environmental effects since it produces millions of gallons of basic and acidic residues, which are then discharged into the environment without any further treatment. As a result of these disparities, microorganisms have been thought of as a replacement source of chitosan because it is even more associated with persistent physicochemical properties.
In recent years, microorganisms have been regarded as an attractive source of chitin and chitosan for industrial applications due to significant advantages. Chitin deacetylase (CDA) is the enzyme that converts chitin to chitosan by the deacetylation of N-acetyl-D-glucosamine residues. The presence of this enzyme activity had also been reported in several other fungi and in some insects. Hence, the two main biological alternatives to the chemical production of chitosan are the fermentation of chitosan containing fungal strains and the use of chitin deacetylases. Therefore, the present study was aimed to isolate, screen and characterize microorganisms for chitosan production.

Materials and Methods
a. Collection of samples
Soil and water samples were collected from different sites such as pond water, canal water,

insect exoskeleton, earthworm gut, vermicompost, garden soil and wheat rhizosphere for the isolation of fungi for chitosan production using chitin agar medium.

b. Isolation of microorganisms for chitosan production

Microorganisms for chitosan production were isolated from soil and water samples by serial dilution and plating on chitin agar medium ($NaNO_3$ 2.0 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 1.0 g/L, $MgSO_4.7H_2O$ 0.5 g/L, Yeast extract 0.5 g/L, Chitin 1.0 g/L, Agar 20.0 g/L and pH 7.0). Two replications were maintained for each of the prepared dilution. After 4-days of incubation at 27 ± 2ºC, colonies of microorganisms were developed on the chitin agar medium and further purified by repeated sub-culturing on Nutrient agar, Knight agar and Potato Dextrose agar media. The isolates capable of growing in chitin agar medium were selected for screening experiments.

c. Screening of isolates for chitin deacetylase activity

The isolates were screened for chitin deacetylase activity by spot inoculating each of the isolates on chitin agar medium with 0.5g/L of p-nitro acetanilide as indicator.

Plate assay

Sterilized chitin agar medium containing p-nitroacetanilide as indicator was poured into sterilized pertiplates. After solidification of the media, spot inoculation of the seventeen fungal isolates by transferring the mycelia on the center of the petri plates were made with the help of flame sterilized loop on each plate aseptically and incubated at room temperature. The pale yellow colour was formed on petri plates with positive isolates. Three replications for each isolate along with an un-inoculated control were maintained.

d. Morphological characterization of isolates

All the positive isolates were sub cultured and incubated at room temperature to study macroscopic characteristics such as colony colour, colony reverse colour and colony edge.

Results and Discussion

a. Isolation of fungi for chitosan production

The fungi for chitosan production were isolated from different sites on chitin agar medium. In total, 18 morphologically different fungal isolates, 17 bacteria and 3 actinomycetes were obtained on chitin agar medium (Table 1).

Table 1: List of isolated microorganisms

Sl. No.	Type of samples	No. of samples	No. of isolates		
			Bacteria	Fungi	Actinomycetes
1.	Pond water	4	2	2	0
2.	Canal water	3	5	3	1
3.	Insect exoskeleton	3	1	3	0
4.	Earthworm gut	3	2	2	0
5.	Vermicompost	2	3	2	0
6.	Garden soil	3	3	4	1
7.	Wheat rhizosphere	3	1	2	1
	Total	21	17	18	3

All the isolates obtained were able to utilize chitin which was confirmed by the growth in selective media. It was also presumed that these isolates where able to produce chitin deacetylase which carry out the hydrolysis of chitin to chitosan thereby generating glucosamine units and acetic acid. This study agrees with earlier findings of Landge *et al.*, (2015) [4] in which bacterial isolates were successfully isolated from soil samples near lake and river. Eighty strains of bacteria were isolated from Langoan hot spring water in North Sulawesi on minimal medium containing Bacto yeast extract, (NH4)2SO4, KH2PO4 and chitin (Toharisman and Suhartono, 2008) [7]. Four species of filamentous fungi, *Aspergillusniger*, *Rhizopusoryzae*, *Lentinusedodes and Pleurotussajo-caju*and two yeast strains, *Zygosaccharomycesrouxii* TISTR5058 and *Candida albicans*TISTR5239 have been reported containing chitin and chitosan in their cell wall and septa (Pochanavanich and Suntornsuk, 2002) [6].

b. Screening of isolates for chitin deacetylase activity

All the isolates obtained were subjected to plate assay by spot inoculating each of the isolates on chitin agar media supplemented with 0.5g/L of p-nitroacetanilide as indicator.

Six isolates of fungi, two bacterial isolates and one actinomycete showed positive results by production of yellow color in the chitin agar media supplemented with p-nitroacetanilide as indicator (Table 2).

Table 2: Screening of isolates for chitin deacetylase activity

Sl. No.	Type of samples	No. of positive isolates with CDA activity		
		Bacteria	Fungi	Actinomycete
1.	Pond water	1	1	-
2.	Canal water	-	1	1
3.	Insect exoskeleton	-	1	-
4.	Earthworm gut	-	-	-
5.	Vermicompost	-	1	-
6.	Garden soil	1	2	-
7.	Wheat rhizosphere	-	-	-
	Total	2	6	1

All the positive isolates were allotted code numbers from FC 1- FC 6 for fungi, BC 1 – BC 2 for bacteria and AC 1 for actinomycete as shown in Table 3.

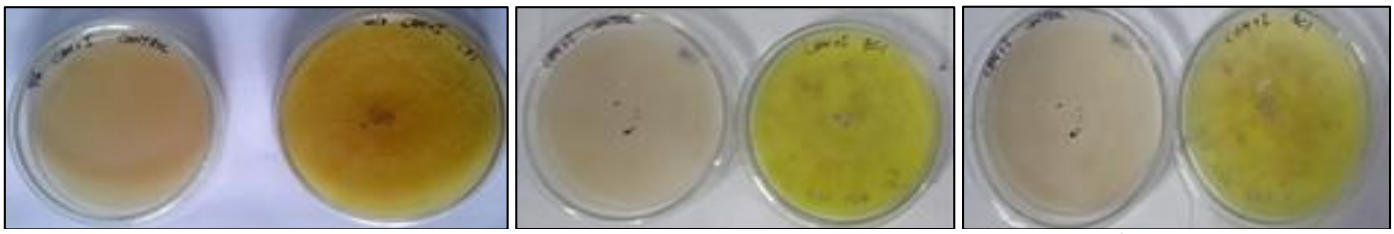
Table 3: List of microorganisms for chitosan production

Sl. No.	Samples	Bacteria	Fungal Isolates	Actinomycete
1.	Pond water	BC 1	FC 1	-
2.	Canal water	-	FC 2	AC 1
3.	Insect exoskeleton	-	FC 3	-
4.	Vermicompost	-	FC 4	-
5.	Garden soil	BC 2	FC 5, FC 6	-

BC – Bacteria for chitosan production, FC - Fungi for chitosan production

AC – Actinomycete for chitosan production

Among these six fungal isolates, two isolates namely FC 2 and FC 3 produced yellow color between 2-4 days of incubation. However, the isolate FC 1, FC 4, FC 5 and FC 6 shown the yellow color between 6-8 days of incubation. The two bacterial isolates (BC 1 and BC 2) as well as one actinomycete (AC 1) took 12 days for color development. The development of yellow color on chitin agar media supplemented with p-nitroacetanilide indicated the presence of chitin deacetylase which was the first evidence of chitosan formation (Plate 1).



Fungi Bacteria Actinomycete

Plate 1: Spot inoculation of Fungi (FC1), bacterial isolates

(BC 1) and actinomycete (AC 1) on chitin agar media

Similar results were reported by Lee *et al*. (2019) [5] who screened 24 bacterial isolates for production of chitin deacetylase enzyme based on the ability of deacetylation of chitin in presence of an artificial substrate p-nitroacetanilide. Karthik *et al.* (2017) [2] reported the production of an extracellular chitin deacetylase (CDA) from *Aspergillus flavus* under solid-substrate fermentation (SSF) using wheat bran as substrate. Related results were also retrieved in studies conducted by Tuli *et al.* (2018) [8] who isolated and screened 142 chitin deacetylase producing microorganisms. Among the 142 microbial isolates, 91 were found to be CDA positive using p-Nitroacetanilide strip and p-Nitroacetanilide agar method. Kaur *et al*. (2012) [3] isolated 20 bacteria from soil samples of different beaches of Chennai, India and screened for their chitin deacetylase activity by using the diagnostic strip test for conversion of p-Nitroacetanilide by the enzyme which was indicated by development of yellow color in the strip.

Morphological characterization of isolates

The positive isolates of fungi were then transferred to Potato Dextrose agar plates to study their colony morphology (Table 4).

Table 4: Morphological characteristics of fungal isolates for chitosan production

Sl. No.	Isolates	Colony morphology		
		Colony Color	Colony Reverse Color	Colony edge
1.	FC 1	Black	Straw colored	Smooth
2.	FC 2	Light green	Cream	Smooth
3.	FC 3	Green with white outer margin	Light orange	Smooth
4.	FC 4	Grey	White	Smooth
5.	FC 5	Dark green	White	Smooth
6.	FC 6	Brownish green	Yellow	Smooth

The morphological characteristic of the fungal isolates showed a variation in colony color from light green to dark green, grey and black. The reverse colony color varied from straw colored to white, orange and yellow. All the fungal isolates showed a presence of smooth colony edges.

Conclusion

In the present study, we have isolated microorganisms for chitosan production from various water and soil samples. Six isolates of fungi, two bacterial isolates and one actinomycete showed positive results for the production of chitin deacetylase. These microorganisms can be used as biological alternative for the conversion of chitin to chitosan.

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