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Isolation and characterization of polyhydroxyalkanoate and exopolysaccharide producing *Bacillus* sp. PS1 isolated from sugarcane field in Bhilai, India

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Abstract

Polyhydroxyalkanoates (PHA) and exopolysaccharide (EPS) are important biopolymers. Bacteria produce polyhydroxyalkanoate by fermenting sugar and lipids as a mechanism of storing carbon and energy under unbalanced growth condition. A bacterium of *Bacillus* group was isolated from sugarcane field in Bhilai which produced PHA and EPS. It was aerobic, Gram positive, rod shaped, endospore forming and catalase producing bacterium. It was able to grow up to 14% NaCl concentration, pH range from 3 to 10 and temperature range from 27°C to 70°C. Maximum yield of PHA was 38.5 mg/ml at 40°C while at 60°C yield was 34.5 mg/ml. Likewise maximum yield of EPS was 18.5 mg/ml at 37°C. The bacterium has other biotechnological aspects also which is evident from the fact that it has catalase activity up to 65°C and produces urease.

Keywords: Bacterium; PHA; Exopolysaccharide,;Catalase; *Bacillus*

Introduction

Microorganisms are important organisms from industrial point of view of which bacteria are most important. Various enzymes like amylase, catalase, cellulase, urease etc. are being produced from bacteria [1-6]. Poly hydroxyalkanoate (PHA) and exopolysaccharide are also produced from bacteria [7-10]. PHAs were seen as the ideal material for replacing petrochemical based plastics. With the development of consciousness about the safety of environment materials which are biodegradable are becoming replacement options of petrochemical based plastics [11].

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Exopolysaccharides are high-molecular-weight polymers, composed of sugar residues. EPS are secreted by microorganisms into their surrounding environment. Exopolysaccharides are extensively used as thickening and gelling agents in a wide range of industrial products and processes [12]. Likewise catalase which breaks hydrogen peroxide into water and oxygen is used for removing pollution caused due to H_2O_2 presence in industrial effluents therefore is an important enzyme isolated from bacteria. PHA and exopolysaccharide producing bacteria have been isolated from soils of sugarcane fields, ammunition polluted soils, effluents of distillery, sewage etc. [13-17].

The present work was undertaken for isolation of PHA and exopolysaccharide producing bacteria from soil and to optimize the medium. The objective of study was to integrate PHA and exopolysaccharide extraction so that exopolysaccharide can be extracted from the broth and PHA from the cells without any loss of both the polymers. The morphological, physiological and biochemical tests were also performed in order to characterize various property of bacterium.

Materials and Methods

Soil from sugarcane field was brought to the laboratory in a presterilized container for isolation of bacteria with prospective PHA producing properties. Soil was air dried for 2 days and bacteria was cultured using serial dilution method on nutrient agar media (NAM) plates.

Culture media

Media used for culture of bacteria from soil was nutrient agar media which contain peptone (5g), beef extract (3g), NaCl (5g), agar (15g), distilled water (1000 ml). For production of PHA and EPS by bacterium 3 different broth medium were used. Broth1 nutrient broth which contain peptone (5g), beef extract (3g), NaCl (5g), distilled water (1000 ml) and broth 2 was Luria broth which while broth 3 contain beef extract (1g), sucrose (5g), NaCl (5g) per liter.

Broth 3 medium was further modified to achieve optimum medium composition for PHA and EPS production. Modified 1 broth 3 medium contain 0.1% Beef extract, 5% Sucrose, 0.5% NaCl, Modified 2 broth 3 medium contain 0.15% Beef extract, 5% Sucrose, 0.5% NaCl , Modified 3 broth 3 medium contain 0.025% Beef extract, 10% Sucrose, 0.5%NaCl, Modified 4 broth 3 medium contain 0.05% Beef extract ,15% Sucrose,0.5%NaCl.

Methods

The bacterium with mucoid colony was isolated and pure cultured in nutrient agar medium at 37°C temperature by serial dilution method for further tests. Various morphological and physiological tests were performed, for which nutrient agar medium was used as culture medium. Colony morphology was determined by growing the bacterium on nutrient agar medium at 37°C temperature. Cell morphology of bacterium growing at 37°C temperature was determined by gram staining and endospore staining. Physiological characterization of bacterium was done by testing the property of the bacterium to be

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able to grow at different temperature (20°C-80°C), pH (3-11) and NaCl Concentration (2%-16%). Then a range of biochemical tests were done to check the biochemical property of bacterium. These tests were catalase, indole, methyl red, VP, citrate utilization, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, urea hydrolysis, nitrate reduction, H2S production, tween80 hydrolysis, O/F, DNase production and litmus test. To observe maximum PHA and EPS production bacterium was cultured at 37°C temperature in 3 different broths medium and in 4 different modification of broth 3. PHA and EPS were also observed in broth 3 medium at 4 different temperatures that was 37°C, 40°C, 50°C, 60°C. For PHA and EPS the bacterium was grown in broth for one week. Instead of two separate methods of isolating PHA and exopolysaccharide the methods to harvest them were integrated.

Method to harvest the polymers

10ml broth containing bacterial cultured was centrifuged for 20minutes at 1500rpm.

The bacterial pellet was taken for PHA extraction and the supernatant for extraction of exopolysaccharide.

Supernatant was again centrifuged for 20minutes at 1500rpm to separate remaining cells. 5ml supernatant was taken in fresh tube and 3 times volume (that was 15ml) ethanol was added to it and was incubated for 1hour to precipitate the exopolysaccharide.

Bacterial pellet was resuspended in 5ml distilled water and lysed by sodium hypochlorite at 37 °C for 1 hour. The lysed mass was centrifuged at 10,000 rpm for 10 minutes and the resulting pellet was washed first with distilled water and then with acetone and alcohol (1:1). PHA was precipitated by addition of chloroform. The PHA recovered after evaporation of chloroform was weighed and 10 µg of it was digested in H_2SO_4 at 100 °C for 10 minutes. It was estimated at 235 nm against crotonic acid as standard. The digested sample was scanned from 190-700 nm against H_2SO_4 standard [3].

Catalase was tested using hydrogen peroxide and production of bubbles was taken as an indication for production of catalase enzyme by the bacterial cells. Presence of activity of catalase was tested by titrating against potassium permanganate and sulphuric acid.

Results

The morphological, physiological and biochemical characters of the bacterium are given in (Table 1, Table 2 and Table 3) respectively. Amount of PHA and exopolysaccharide produced by the bacterium in different media are given in (Table 4). In (Table 5) the amount of PHA and exopolysaccharide produced by the bacterium in different modification of broth 3 is given. There is also variation in amount of PHA and exopolysaccharide produced by the bacterium at different temperatures which is given in Table 6. The highest amount of PHA produced was 38.5 mg/ml at 40°C and 34.5 mg/ml at 60°C. Only one major peak was visible at 340 nm which indicated that only one type of

S.No.	Test	Results
1. Colony Morphology		
A	Configuration	Circular
B	Margin	Wavy
C	Elevation	Elevated
D	Pigment	-
E	Opacity	Opaque
F	Surface	Smooth
2. Cell Morphology		
A	Gram Stain	Positive
B	Cell Shape	Rods
C	Arrangement	Single, Group, Two cell, Chains
D	Endospores	Present
E	Motility	-

Table 1: Results of Morphological tests performed on the bacterial strain at 37°C temperature.

S.No.	Growth Condition	Results
1. Growth at different temperature		
A	20°C	-
B	25°C	-
C	27°C	+
D	30°C	+
E	37°C	+
F	47°C	+
G	57°C	+
H	60°C	+
I	67°C	+
J	70°C	+
K	77°C	+
L	80°C	-
2. Growth at different pH		
A	3	+
B	4	+
C	5	+
D	6	+
E	7	+
F	8	+
G	9	+
H	10	+
I	11	+
3. Growth at different conc. Of NaCl		
A	2%	+
B	4%	+
C	6%	+
D	8%	+
E	10%	+
F	12%	+
G	14%	+
H	16%	-

Table 2: Results of physiological tests performed on the bacterial strain at various growth conditions.

S. No.	Test	Results
1	Catalase	+
2	Indole	-
3	Methyl Red	-
4	VP	+
5	Citrate utilization	+
6	Casein hydrolysis	+
7	Gelatin hydrolysis	-
8	Starch hydrolysis	+
9	Urea hydrolysis	+
10	Nitrate reduction	+
11	H2S production	-
12	Tween 80 hydrolysis	+
13	O/F	O/F
14	DNase production	-
15	Litmus test	Proteolysis

Table 3: Results of Biochemical tests performed on the bacterial strain.

PHA was produced. Highest amount of EPS produced was 18.5 mg/ml at 37°C. Catalase activity revealed that it is active up to 65°C and there is no difference in the amount of activity shown at different temperatures (37°C, 40°C, 50°C, 60°C and 65°C) .

Discussion

The bacterium isolated from the soil of sugarcane in Bhilai is proposed to be a member of *Bacillus* group on the basis of its morphological and biochemical tests. The bacterium is rod shaped, endospore producing, and catalase positive and aerobic and on these criteria it can be placed in the *Bacillus* group. The physiological properties of the bacterium is remarkable as it able to grow at pH 3-pH 10. It is also able to grow at NaCl concentrations up to 14%. The most remarkable physiological property of the bacterium is the ability to grow at wide range of temperatures. This is also shown by the catalase activity as the enzyme shows no difference in its activity from 37°C to 77°C. PHA is produced by the bacterium and its production was tested at different temperatures. Likewise exopolysaccharide production was also tested at the same temperatures.

The change in temperature changes the amount of PHA and EPS production. PHA production increased as the temperatures increased while the amount of exopolysaccharide decreased with the increase in temperature. PHA production was highest at 60°C in broth 3. Similar quantity was obtained of PHA and exopolysaccharides from samples which were processed separately from those samples where PHA and exopolysaccharides were obtained in a single sample (Table 7). With the increase in temperatures the amount of PHA production also increased and at lower temperatures EPS production was favorable. Various other workers have given the amount of PHA produced in terms of dry weight which we are avoiding as the work done here is for optimizing the medium for PHA production [15-16].

S. No.	BROTH	Results	
		PHA (mg/ml)	EPS (mg/ml)
1.	Broth 1	12.8	8.5
2.	Broth 2	8.5	10.0
3.	Broth 3	20	18.5

Table 4: Amount of PHA and EPS produced by bacterium in different medium at 37 °C temperature.

S.No.	Modified Broth 3	Results	
		PHA (mg/ml)	EPS (mg/ml)
1	Modified :1	7.7	7.1
2	Modified :2	38.5	-
3	Modified :3	5.7	2.1
4	Modified :4	5.7	7.1

Table 5: Amount of PHA and EPS produced by bacterium in different modified broth 3 medium at 40 °C temperature.

S. No.	Temperature	Result	
		PHA (mg/ml)	EPS (mg/ml)
1	37°C	20	18.5
2	40°C	24.5	12
3	50°C	27.3	8
4	60°C	34.5	2

Table 6: Production of PHA and EPS in broth 3 medium at different temperatures.

S. No.	Method	PHA (mg/ml)	EPS (mg/ml)
1	Only PHA	25	-
2	Only EPS	-	12.3
3	PHA and EPS both from same sample	24.3	12

Table 7: Amount of PHA and EPS from different methods.

The results show that with the bacteria producing both PHAs and exopolysaccharides both can be obtained from the same sample without loss of quantity of both polymers. This will reduce time and labour in the extraction method.

Conclusion

It can be concluded from the above study that PHA and exopolysaccharide producing bacteria can be isolated from sugarcane field. Production of PHA is higher than the EPS yield will be lower and vice versa. The results also show that at higher temperatures PHA production is favored and at lower temperatures EPS production is favored.

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