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PHB PRODUCTION USING NOVEL AGRO-INDUSTRIAL SOURCES FROM DIFFERENT *BACILLUS* SPECIES.

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ABSTRACT

For the production of polyhydroxybutyrate (PHB) different *Bacillus* species were isolated from soil samples. After the morphological and biochemical studies, isolates were identified as *Bacillus cereus, Bacillus subtilis, and Bacillus megaterium*. These *Bacillus* species were further explored for their potential to produce PHB using different low cost agro-industrial materials. PHB production was studied using agro-industrial materials like Jawar stem, Neera, Cashew apple pulp, Sugar cane bagasse, Coconut pulp and Grapes pulp. Extraction of PHB was done by hot chloroform method. PHB production was quantified using crotonic acid assay. Highest cellular PHB content was obtained from *Bacillus subtilis* with Neera as source of carbon which was found to be 0.284g/L. As Neera is a natural drink having very less shelf- life and majorly used for drinking purpose, its use in PHB production may prove beneficial, and hence may become an area of further research.

KEYWORDS

PHB, Bacillus sp., Agro industrial waste, Neera, Crotonic acid.

INTRODUCTION

Now a days management of plastic waste is a major worldwide concern. As a synthetic plastic is non-biodegradable, it is creating a great havoc to environment. Plastic and synthetic polymers are mainly produced using petrochemical material and they contribute to environmental pollution .They are also dangerous to number of animal species[1]. Although plastic has many advantages its non-biodegradability is a major drawback, which forced us to think upon a material which can replace plastic. Earlier studies showed that Polyhydroxybutyrate (PHB) is bio-degradable material, which has physical properties similar to the synthetic plastic. PHB has various applications in different areas like medicine, drug manufacture, agriculture and various industrial purposes. Some of the major applications of PHB are in drug delivery in developing medical sutures, bone marrow scaffold, tissue engineering devices and agriculture products[2]. PHB is equivalent to a very basic polymer, PHA (polyhydroxyalkanoates) [1]. PHB is produced by microorganisms in the form of reserved food granules under stress condition like excess availability of carbon source but limited provision of other nutrients such as nitrogen, phosphate, oxygen and sulphur. PHB was first reported from *Bacillus megaterium* by Lemoigne[3], similarly in 2006 Valappil *et al* identified and characterized *Bacillus cereus* that produces large amount of PHB. Many references show that number of microorganisms like *Alcaligene eutrophus, Azotobacter beijierinckia, Pseudomonas oleovorans* etc. produce PHAs as reserve food material. The main limitation in commercial production of PHB as biodegradable plastic is its high production cost as compared to that of production of synthetic plastics based on petrochemicals. Low cost agro industrial materials can be used to economize the production.

MATERIAL & METHODS

Agro Industrial Sources:-

In routine practice, jawar stem is a waste product after harvesting Jawar crop (*Sorghum bicolor*).This waste is usually used as cattle feed or as fuel in rural areas. As it contains moderate amount of sugar, it is used in the present study as a crude carbon source for PHB production.

Neera is used as natural drink and also in preparation of several food products. Neera is known to contain high sugar content. It is extracted from inflorescence of Toddy plant (*Borassus flabellifer)* and sold at very cheap rates on 'Neera Vikri Kendra' in India. Because of high-sugar content, low-cost and availability Neera is used as source of carbon in the study.

Cashew apple is a pseudo fruit of cashew plant (*Anacardium occidentale*).This also has moderate sugar content. Every year huge amount of cashew apple goes waste after harvesting cashew nut in India.

Sugarcane bagasse is waste from sugar factories that goes to paper industry for paper production or used as fuel in rural areas. As it contains high sugar concentration it is used as carbon source for the production of PHB in the present study.

Grapes waste and coconut pulp also have moderate sugar concentrations, which can be used as carbon source for PHB production.

Producer species:

Organisms like gram positive bacteria grow rapidly, they possess various hydrolytic enzymes and produce co-polymers from structurally unrelated sources, and hence they prove to be ideal candidates for PHB production on industrial scale [4].

Isolation & Identification

Soil samples were obtained from different areas around Pune city, where soil was contaminated with oil. One gram of soil from each sample was suspended in 99 ml of sterile saline water and shaken vigorously for 2 minutes. Suspension was serially diluted from 10^{-3} to 10^{-7} and was plated on nutrient agar plates. The plates were incubated at 37^0C for 24 to 48 hrs. Isolated colonies were sub-cultured till the isolated single colony was obtained and preserved on nutrient agar slant at 4°C. The isolates producing PHB were identified by various biochemical tests like colony morphology, Gram staining, motility test. For determination of carbon source utilization sugars like glucose, sucrose, arabinose, lactose, xylose, and mannitol were used. Tests like starch hydrolysis, nitrate reduction, lecithinase production test, casein hydrolysis test were performed for identification [1, 5].

Screening for PHB production by Bacillus

PHB production by *Bacillus* was screened by Sudan black test[1] (Figure-1). *Bacillus* was grown on nutrient agar as a single colony. After 48 hrs of incubation, approximately 8 ml Sudan black solution (0.2% Sudan black B in 96% ethanol) was added to the plate and incubated for 10 min. After that the dye was decanted and plates were gently rinsed with 10 ml 100% ethanol. Colony stained with Sudan black B were selected as a positive for PHB production[1].

The PHB producing isolates were identified by 16S r RNA analysis (NCCS, PUNE).

Analytical method

One ml of 24 hour old culture was inoculated in 9 ml nutrient broth (peptone 5 g/l ,NaCl 5 g/l ,Beef extract 1.5 g/l , yeast extract 1.5 g/l) and 9 ml yeast extract glucose media (yeast extract 1 g/l ,glucose 10 g/l,dipotassium phosphate 0.5g/l, magnesium sulphate 0.2g/l ,sodium chloride 0.1 g/l) separately. These were used as inoculums after growing it for 24 hrs and added to 100ml of the same media. Flasks were incubated at 37^0C for 48 -72 hours on shaker incubator at 170 rpm.

Use of different agro-industrial sources

Neera was procured from local Neera stall, Cashew fruits were bought from Ratnagiri local market, Jawar stem were obtained from a farm nearby Pune city, and sugarcane bagasse was procured from local sugar mill. Grapes waste and coconut pulp were obtained from MITCON food processing unit, Pune.

Diluted acid hydrolysis of solid waste

PHB production by *Bacillus* species was tested using hydrolyzed sources, except Neera and grapes waste. Neera was filter sterilized and grapes waste was filtered and autoclaved separately.

All other sources viz; jawar stem, Cashew fruit, sugarcane bagasse, coconut pulp were hydrolyzed by 0.5-5% v/v sulphuric acid (solid: liquid (1:10-1:20)[6] and autoclaved at 121°C, and 15 psi for 30 min. The hydrolyzed samples were filtered and supernatants were neutralized using sodium hydroxide (6N). Reducing sugar content was determined (DNSA method)[4, 7]. Media were prepared using these hydrolysates. After autoclaving, 10% v/v, 24 hrs old cultures of *Bacillus* species were inoculated in the modified media and incubated at room temperature for 48 to 72 hrs on shaker incubator[4].

PHB extraction

After incubation each sample was centrifuged for 15 minutes at 6000 rpm. Pellet was washed twice with sterile deionized water and dried for

24 hrs at 100°C.The total bacterial dry weight was determined. Sodium hypochlorite was added to dry cell biomass and was incubated at 60°C for 1 hour to break the cell wall of bacteria. This sample was centrifuge at 6000 rpm for 15 minutes and supernatant was used for further treatment. Using 96% v/v ethanol: acetone (1:1) cell lipid and other molecules, except PHB were extracted from supernatant. The PHB extraction was done by hot chloroform method (adding chloroform to the tube containing supernatant in water bath). PHB crystals were obtained after evaporation of chloroform [2, 5](Figure-2).

Determination of PHB content

PHB crystals undergo dehydration on treatment with sulphuric acid and heat, to crotonic acid. The extracted PHB was converted to crotonic acid by adding 98% sulphuric acid and heating to 60 °C for 1 hr. Crotonic acid shows maximum absorption at 235nm.The absorbance of the solution was measured at 235 nm in a UV spectrophotometer (Systronics UV-VIS spectrophotometer 117) against a sulphuric acid as blank. The amount of PHB per gram dry weight of bacterial cells was determined from standard graph of crotonic acid[2, 5].

RESULTS AND DISCUSSION

Sudan black staining showed ability of *Bacillus* to produce PHB. For all the isolates, biochemical tests were done as per Bergey's Manual and further confirmation was done by 16S rRNA analysis. The 3 isolates were identified as *Bacillus cereus, Bacillus subtilis, and Bacillus megaterium*. Quantitative estimation of PHB content revealed that Bacillus *cereus, Bacillus subtilis, Bacillus megaterium* can accumulate about 0.04 gm/l, 0.1 gm/l and 0.08 gm/l of dry weight as PHB using nutrient broth supplemented with sugar like 1% glucose.

In this study, we have used six agro-industrial materials as feed stocks as Neera, Jawar stem, Cashew apple, Sugarcane bagasse, grapes pulp and coconut pulp. In order to quantitate sugar content of these low cost sources, DNSA test was performed. *Bacillus cereus* and *Bacillus subtilis* were studied for accumulation of PHB using Neera, Jawar stem and Cashew apple.PHB yields obtained are as per Table-1.

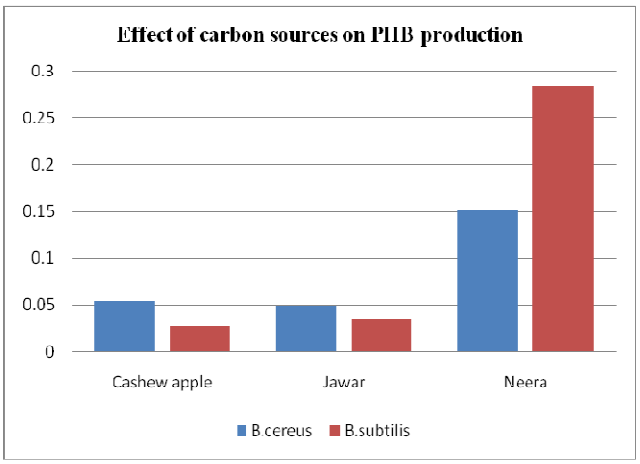
It was observed that Neera was best feedstock among the other crude source used. *Bacillus subtilis* showed highest production of PHB using Neera (0.284 g/l) and *Bacillus cereus* gave 0.152 g/l PHB using Neera. *Bacillus subtilis* gave lowest yield with Cashew apple as a carbon source and Bacillus *cereus* with Jawar stem which were found to be 0.027g/l and 0.034g/l respectively. Simultaneously *Bacillus megaterium* was explored for production of PHB using Sugarcane bagasse, Grapes pulp and Coconut pulp. With Sugarcane bagasse as a carbon source, *Bacillus megaterium* gave 0.199g/l PHB dry weight. Grapes pulp did not give prominent PHB yield (0.006 g/l).

Table-1
PHB production by Bacillus species

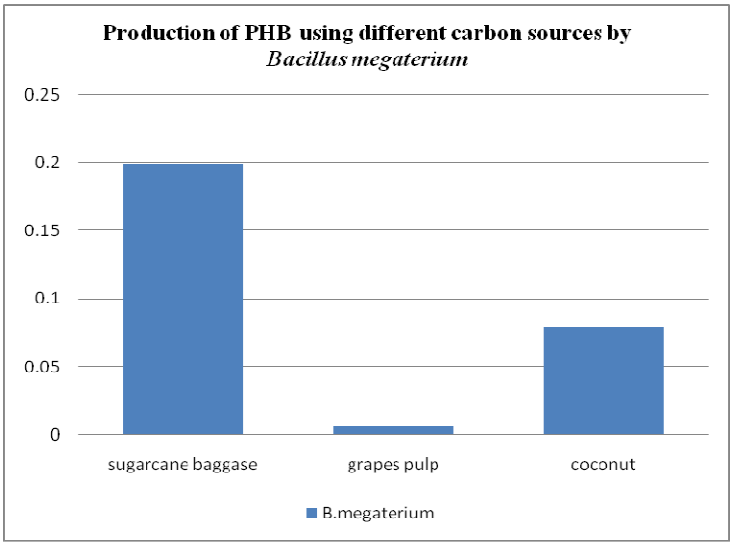
Agro industrial sources	Sugar concentration (mg/ml)	Organisms	Cell biomass(dry weight)[gm/l]	*PHB [gm/l]
Cashew apple	0.22	*Bacillus subtilis*	4.92	0.027
		Bacillus cereus	5.01	0.054
Jawar stem	1.9	*Bacillus subtilis*	8.56	0.034
		Bacillus cereus	6.72	0.049
Neera	2.52	*Bacillus subtilis*	6.440	0.284
		Bacillus cereus	4.448	0.152
Sugarcane bagasse	3.17	*Bacillus megaterium*	2.257	0.199
Grapes pulp	0.12		2.028	0.006
Coconut pulp	2.01		2.058	0.079

(*Dry PHB weight per dry cell biomass)

Graph - 1
PHB production by B.cereus and B.subtilis using different agro-industrial sources



Graph - 2
PHB production by Bacillus megaterium



Graph – 3
Comparision of Dry cell mass and PHB production by Bacillus species

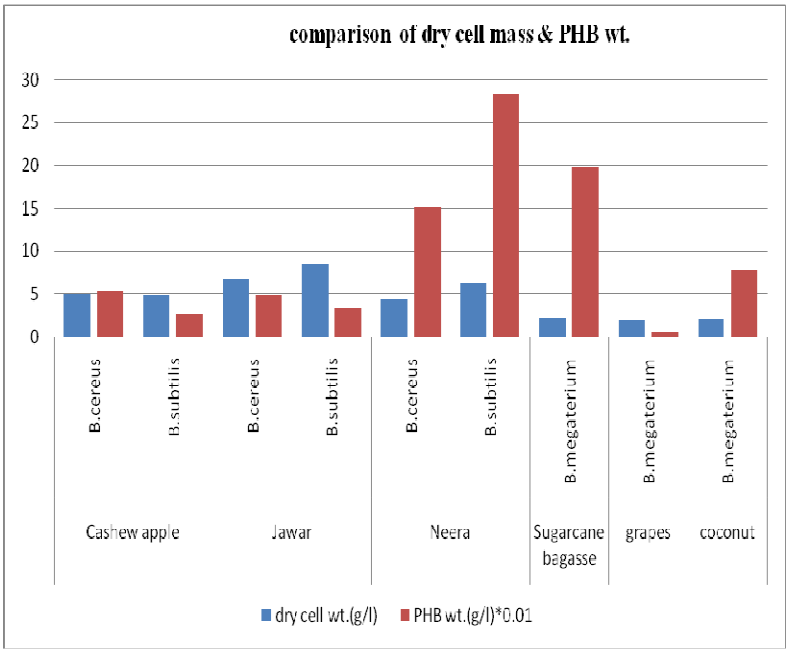


Figure – 1
Sudan black staining of isolated Bacillus spp.

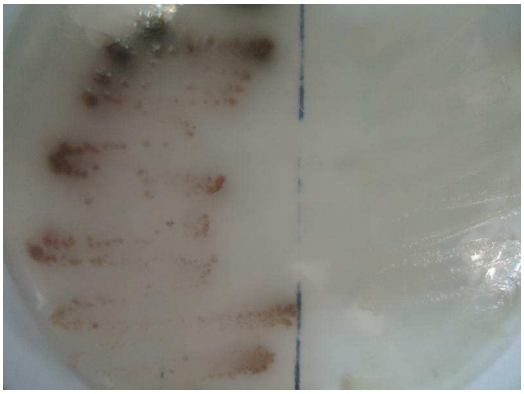
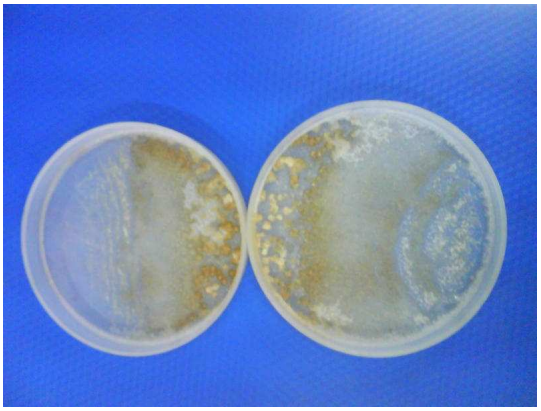


Figure – 2
PHB crystals after purification



CONCLUSION

Bacillus cereus, Bacillus subtilis and *Bacillus megaterium* can utilise a wide variety of agro-industrial materials for PHB production. This can be exploited for the production of PHB at commercial level. Utilisation of agroi-ndustrial materials in production of biodegradable plastic (PHB) will not only ensure the low production cost but also solve the problem of managment of waste material to a certain level.

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