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Production and isolation of polyhydroxyalkanoates from *Haloarcula marismortui* MTCC 1596 using cost effective osmotic lysis methodology

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are completely biodegradable and biocompatible plastics having interesting characteristics for a significant number of industrial applications. The production amount of these polymers at the industrial scale is however minimal and their cost of production is higher. This is mainly because of the high economic need of traditional solvent extraction based downstream strategies of PHAs isolation. In this research work, we have studied the production and isolation of polyhydroxyalkanoates from halophilic archaebacterial strain, Haloarcula marismortui MTCC 1596. The strain has produced high amounts of PHAs in the presence of excess carbon and limited nitrogen source in the medium. The intracellular PHA compounds were isolated by osmotic cell lysis method using distilled water. This method was found to be simple and cost effective for cell lysis and it can be able to reduce the high downstream processing expenditure of conventional PHAs isolation procedure. Further, the optimisation of PHAs production media was carried out by altering the carbon sources. Among all, sodium acetate was found to be a potential source for the high production of PHAs. Through the results we obtained, it can be concluded that the production and isolation of polyhydroxyalkanoates from this halophilic archaeabacterial strain using osmotic cell lysis method is profitable.

Keywords: Polyhydroxyalkanoates, Bioplastics, *Haloarcula marismortui*, Osmotic cell lysis.

INTRODUCTION

Bioplastics are biodegradable, compostable and bioerodible plastics. Among the various kinds of bioplastics, polyhydroxyalkanoates (PHAs), a form of microbially synthesised biodegradable polyesters are showing good promises for the future commercial mass production of biodegradable plastics. These PHAs are natural biopolymers, which are synthesised and catabolised by various microorganisms and have certain advantages over petroleum-derived plastics [1]. They can be produced from renewable resources, which are recyclable and

considered as natural materials. These properties make PHAs an appropriate substitution to petrochemical thermoplastics [2]. They are structurally simple macromolecules synthesised by many prokaryotic microorganisms such as eubacteria, archaebacteria and cyanobacteria [1]. PHAs have applications in perspective areas like; the use of PHAs as filler for non-biodegradable plastics, disposable packages, in agriculture - systems for prolonged release of fertilisers and agrochemicals, in medicine -medical devices and systems of sustained drug delivery [3]. PHA biopolymers promise to have a significant role in tissue engineering and the development of living tissue products for therapeutic applications [4]. Although wide range of microbial populations are available to produce PHAs using cost effective substrates, certain problems are prevailing with the industrial production of polyhydroxyalkanoates and these hurdles make their role smaller comparing with other biodegradable polymers and synthetic plastics [5]. One such major hurdle lies with the cost of PHAs production. The price of PHAs is found to be four to nine times higher than that of synthetic plastics, which has hindered the demand for these biodegradable polymers [6]. Therefore, more efficient and cost-effective technologies need to be developed to make PHAs cost competitive in relation to products with similar applications. This current research focuses on a cost effective osmotic lysis based downstream methodology for extraction of intracellular PHAs produced by halophilic archaebacteria.

MATERIALS AND METHODS

Materials:

Haloarcula marismortui MTCC 1596 was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. Crotonic acid was obtained from SRL chemicals, Mumbai. All other chemicals and reagents used in the study were procured from Merck chemicals, India.

Table 1: Nutrient Rich Medium

Constituents	g/l
Casamino Acids	5
Yeast extract	5
Sodium glutamate	1
Tri sodium citrate di hydrate	3
Sodium chloride	200
Magnesium sulphate hepta hydrate	20
Potassium chloride	2
Ferrous sulphate hepta hydrate	0.36
Manganese chloride tetra hydrate	0.00036
pH	7.2

Table 2: Nutrient Deficient Medium

Constituents	g/l
Sodium chloride	200
Magnesium sulphate hepta hydrate	20
Potassium chloride	2
Sodium glutamate	1
Potassium dihydrogen phosphate	0.0375
Ferrous sulphatehepta hydrate	0.05
Manganese chloride tetra hydrate	0.00036
Yeast extract	1
pH	7.2

Production of PHAs:
3 ml of stock culture was inoculated in 50 ml of sterile nutrient rich medium (NRM) (Table 1) [7] under aseptic conditions and incubated at 37ºC in a shaker incubator for 72 h. After 72 h, 3 ml of the culture from nutrient rich medium was transferred to sterile nutrient deficient medium (NDM) (Table 2) [7] under aseptic conditions and incubated at 37ºC in a shaker incubator. After 5 days of incubation, the cultures were harvested and subjected to further experiments for detection, isolation and estimation of the polyhydroxyalkanoates.

Detection of PHA granules by Sudan black staining:
A loopful of culture was taken and mixed with 20% brine and smeared on a clean glass slide. The smear was air dried and fixed by immersing in 2% acetic acid for 5 min. The slide was then removed, dried, covered with Sudan black solution (0.3 g of Sudan black in 100 ml of Ethanol) and kept at room temperature for 15 min. The excess stain was drained off; the slide was dried by blotting thoroughly and then cleared by adding Xylene (xylol). The slide was blotted and 0.5% aqueous Safranin was used as a counter stain and kept for 10 s. The slide was then washed with distilled water, blotted, dried and examined under light microscope [8].

Isolation of polyhydroxyalkanoates by osmotic cell lysis methodology:
The broth containing halo archebacterial cells were subjected to centrifugation at 10000 rpm for 15 min. The pellet was suspended in distilled water at 5°C for 24 h. This condition ensures the lysis of cells. This lysed suspension was centrifuged at 5000 rpm for 30 min. The pellet was washed 5–10 times with the same volume of distilled water. Finally, a pure white substance was obtained. This indicates that, there were almost no cells or cell membranes left since they have freed from pink colour. The final pellet was dried in an oven at 80°C until a constant weight was attained. The white dust resulting from this treatment was dissolved in chloroform. Most of the material dissolved readily and the undissolved remains were removed by filtration. Finally, the chloroform was evaporated at room temperature and a thin film of polyhydroxyalkanoates was obtained. This film was then characterised by further spectroscopic analysis [9].

UV-Vis Spectroscopic analysis of PHA:
The principle of this assay is to dissolve the samples isolated from bacterial culture in a suitable solvent and subjecting it to scanning in a UV-Vis spectrophotometer under a prescribed wavelength range. The PHA compounds obtained from cultures by osmotic lysis extraction method and was then dissolved in distilled chloroform and subjected to scanning in UV-Vis spectrophotometer (Shimadzu) in the range of 800–200 nm against chloroform blank and the spectrum was then analysed [10].

Estimation of PHAs by crotonic acid assay:
The amount of PHAs in a sample taken can be determined by spectrophotometric assay. This assay was facilitated by the conversion of PHAs into crotonic acid by sulphuric acid treatment. Crotonic acid standard solution was prepared with different increasing concentrations (10 to 40 µg). Absorbance of crotonic acid was measured at 230 nm and a standard curve was plotted. Then, the sample containing 5 to 50 µg polymer in chloroform was taken in a clean test tube and the solvent was evaporated by heating in a boiling water bath. Then, 10 ml of concentrated H_2SO_4 was added to the tube and heated for 10 min at 100°C in a water bath. The solution was then cooled and thoroughly mixed. The sample was then transferred to a quartz cuvette and the absorbance was measured at 230 nm against a sulphuric acid blank [11]. The amount of crotonic acid was then calculated by plotting graph.

Media optimisation for PHAs production:

Different nutrient limited media with different carbon sources and marine salt composition were prepared (Table 3) and inoculated each with 3 ml of culture grown in nutrient rich medium and incubated at 37°C in shaker incubator for 5 days. Then, the culture was harvested and the growth was calculated by dry weight determination and the amount of PHAs produced was estimated by using crotonic acid assay. The following table shows different media involved in the study and their composition.

Table 3: Media design for optimum production of PHAs (Modified from Han et al., [7])

CONSTITUENTS	NDM-1 (g/l)	NDM-2 (g/l)	NDM-3 (g/l)
Sodium chloride	200	200	200
Magnesium sulphate hepta hydrate	20	20	20
Potassium chloride	2	2	2
Sodium glutamate	1	1	1
Yeast extract	1	1	1
Calcium chloride di hydrate	-	-	-
Sodium bromide	-	-	-
Ferrous sulphate hepta hydrate	0.05	0.05	0.05
Manganese chloride tetra hydrate	0.00036	0.00036	0.00036
Potassium di hydrogen phosphate	0.0375	0.0375	0.0375
Peptone	-	-	-
Glucose	0%	1%	-
Sodium acetate	-	-	1%
Distilled water	1000 ml	1000 ml	1000 ml
pH	7.2	7.2	7.2

RESULTS AND DISCUSSION

Polyhydroxyalkanoates are completely biodegradable and biocompatible plastics having interesting characteristics for a significant number of industrial applications but the amount of production of these polymers at the industrial scale is minimal and their cost of production is higher than that of synthetic plastics [12]. The main reason behind the high cost of bioplastic production is its downstream strategies, for the isolation of PHA compounds. We studied the production and isolation of polyhydroxyalkanoates from the halophilic archaebacterial strain, *Haloarcula marismortui.*

Sudan black staining of PHA granules:

The *H. marismortui* cells grown in nutrient rich medium and nutrient deficient medium were subjected to Sudan black staining for the detection of PHA granules. The slides were observed under light microscope. Black stained granules with a background of pink were observed confirming the presence of PHAs [13]. Accumulations of PHA granules were observed in the cells grown on both the nutrient conditions but the number of stained granules was extremely high in the cells grown on nutrient deficient condition.

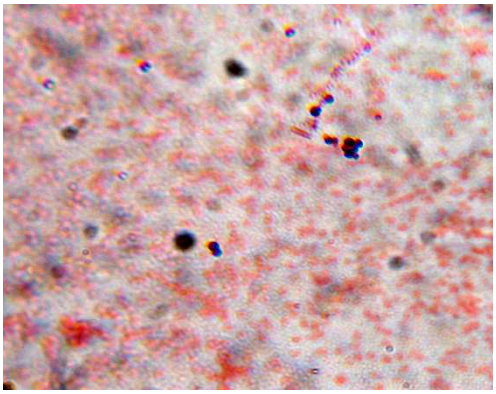

Figure 1: Cells of *H. marismortui* stained with Sudan black and Safranin grown in NRM

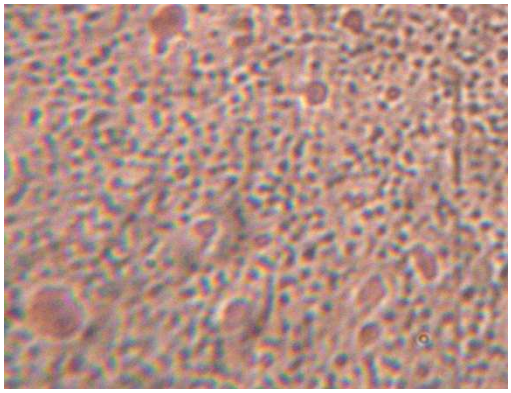

Figure 2: Cells of *H. marismortui* stained with Sudan black and Safranin grown in NDM

Isolation and estimation of PHAs:

The PHAs are usually isolated from microbial cells using solvent extraction methodology which involves large amount of chemical solvents and thereby, increases the cost of PHAs and also load drastic chemical vapours in the environment. In this research work, the PHAs produced by *H. marismortui* cells were extracted by osmotic cell lysis methodology [9]. The cells grown in nutrient deficient medium were harvested and subjected to osmotic lysis. The halophilic bacteria grown on high salt concentration were lysed in the hypotonic condition provided by the normal distilled water and this facilitates the release of PHAs. The isolated PHAs were then dissolved in chloroform and subjected to UV-Vis scanning over the range of 200–800 nm. The result thus obtained, has shown a sharp peak and absorbance at 240 nm (Figure 3). But in solvent control there was no such absorbance at 240 nm. This clearly indicated the presence of PHA compounds in the isolated sample [13].

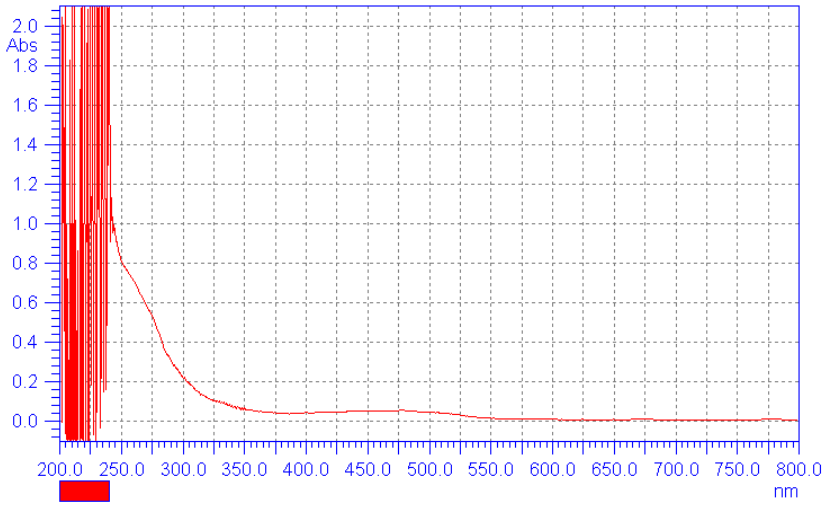

Figure 3: UV-Visible scanning spectrum of PHA compounds isolated from *H. marismortui*

Estimation of PHAs by crotonic acid assay:

The isolated PHAs from *H. marismortui* cultures were estimated by crotonic acid assay. The method was proposed by Lawand Slepecky, in which the PHA samples were treated by heating with concentrated sulphuric acid and converted to crotonic acid which was then analysed in UV-Vis spectrophotometer [11]. The absorbance for the concentrated H_2SO_4 treated PHA samples was taken at 230 nm. The corresponding concentration of crotonic acid was estimated by extrapolating the standard curve and it was found to be 105 µg (per ml) for the sample obtained from *H. marismortui*.

Media design for optimum production of PHAs:

Many microorganisms were found to produce PHAs under certain stress conditions such as limitations of nitrogen, phosphorous or sulphate or lower concentration of oxygen or higher C:N concentration in the feed substrate [14]. In this research work, we analysed media compositions with varying carbon sources such as 0% and 1% of glucose and 1% of sodium acetate. Among different experimental conditions analysed, a good mass of PHAs yield was obtained when sodium acetate was used as carbon source (NDM-3). This identifies the Sodium acetate to be a good substrate for PHAs accumulation in microorganisms [15]. It was also observed that *H. marismortui* was able to grow in all different salt concentrations tested [Unpublished data]. The cells grown in media with our carbon source (NDM 1) have produced very less amount of PHAs on comparing to other experiments. This proves the essentiality of C:N concentration in the PHAs production [16]. However, *H. marismortui* was found to accumulate PHAs under any nutrient deficient conditions experimented (Figure 4).

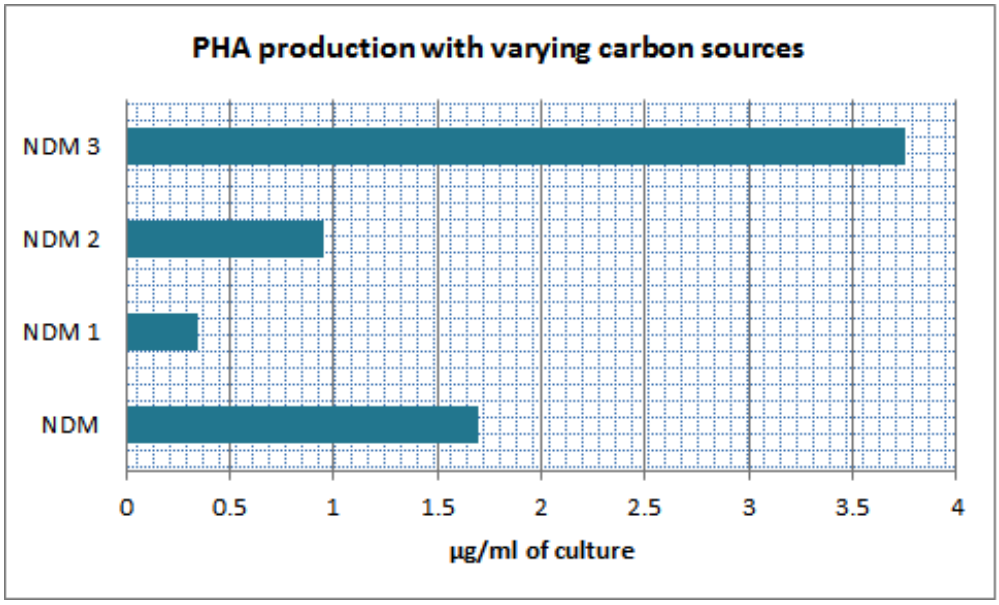

Figure 4: Growth measurement of *H. marismortui* grown in nutrient deficient media

The study was also extended to analyse the byproducts of polyhydroxyalkanoates production from *H. marismortui*. It was found that the strain was capable of producing halophilic protease and rhodopsin pigment [Unpublished data].

CONCLUSION

The current research work with the halophilic archaeabacterial strain, *Haloarcula marismortui* MTCC 1596 for the production of polyhydroxyalkanoates identifies a simpler and effective methodology to extract polyhydroxyalkanoates and also a viable halophilic strain for the production of the same. The content of the research is highly motivated towards the industrial relevancy and thus, it can create a non negligible impact on economics of PHAs production if concerned and researched further with more additional dynamic parameters.

REFERENCES

[1] A. Steinbuchel, H. E. Valentin, *FEMS Microbiol. Lett.*, **1995**, 128, 219–228.
[2] Y. Poirier, D. E. Dennis, K. Klomparens, C. Somerville, *Science.*, **1992**, 256, 520–523.
[3] K. Sudesh, H. Abe, Y. Doi, *Prog. Polym. Sci.*, **2000**, 25, 1503.
[4] B. E. Chaignaud, R. Langer, J. P. Vacanti, In: A. Atala, D. Mooney (Eds.), Synthetic Biodegradable Polymer Scaffolds (Birkha¨user, Boston, **1997**).
[5] G. Braunegg, B. Sonnleitner, R. M. Laffer, *Eur. J. Appl. Microbiol. Biotechnol.*, **1978**, 6, 29–37.
[6] C. S. Reddy, R. Ghai, R. Kalia, *Bioresour. Technol.*, 2003, 87, 137–146.
[7] J. Han, Q. Lu, L. Zhou, J. Zhou, H. Xiang, *Appl. Environ. Microbiol.*, **2007**, 73, 6058–6065.
[8] K. L. Burdon, *J. Bact.*, **1946**, 52, 665–678.
[9] F. R. Castillo, R. F. Valera, G. J. Ramos, R. F. Berraquero, *Appl. Environ. biotechnol.*, **1986**, 51, 214–216.
[10] J. S. Poindexter, L. F. Eley, *J. Microbial. meth.*, **1983** 1, 1–17.
[11] J. H. Law, R. A. Slepecky, *J. Bact.*, **1960**, 82, 33–36.

[12] D. Abd-El-haleem, A. Amara, S. Zaki, A. Abulhamd, G. Abulreesh, *Int. J. Environ. Sci. Tech.*, **2007**, 4 (4), 513–520.
[13] Amutha Santhanam, Sreenivasan Sasidharan, *Afr. J. Biotechnol*., **2010**, 9(21), 3144–3150.
[14] R. M. Lafferty, B. Korstako, W. Korstako, In: H. J. Rehm, G. Reed (Eds.), Microbial production of poly-hydroxybutyric acid, Biotechnology (VCH, Weinheim, **1988**) 136–176.
[15] M. Sharifzadeh Baei, G. D. Najafpour, H. Younesi, F. Tabandeh, H. Eisazade, *World. Appl. Sci J.*, **2009**, 7 (2), 157–161.
[16] Sangkharak K, Prasertsan P. *Electronic Journal of Biotechnology*, **2008**, 11(3).