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Research Paper

Isolation, Characterization, Optimization and Purification of Phb Depolymerase

BIOLOGY

KEYWORDS : PHB, PHB depolymerase, extracellular

ARPITABEN TULSIBHAI PATEL

Research Scholar (M.Sc., M.Ed.), P.G.Department of Education, S.P.University, V.V.Nagar. Gujarat, India

ABSTRACT

The screening and isolation of PHB degrading microorganisms were done on the basis of the ability to produce extracellular PHB depolymerase on PHB containing BHM agar plates. Those isolates were selected which were produced clear zone on PHB agar plate due to hydrolysis of PHB. The optimal condition for PHB depolymerase production was found to be at 30 ℃ and pH 6 for fungal culture, initial substrate concentration of 0.2%(w/v). Higher % Weight loss of polymers was observed in case of shaking condition (12%) than static condition (7%). MP5 showed maximum PHB depolymerase production of 2.88 U/ml at 30°C and pH 6 with substrate concentration of 0.2%(w/v).

INTRODUCTION

Plastics are man-made long chain polymeric molecules. They are widely used, economical materials characterized by excellent all-round properties, easy molding and manufacturing. Traditionally plastics are very stable and not readily degraded in the ambient environment. As a result, environmental pollution from synthetic plastics have been recognized as a major problem. Biodegradation may lead to complete removal of the material from the environment. PHA is polyesters of various hydroxyalkanoates that are synthesized by many organisms at least 75 different genera. These biopolymers accumulate as distinct 0.2-0.7 µm diameter granular inclusion bodies in response to nutrient limitation, especially in pseudomonas. The most widely encountered PHAs are poly β-hydroxybuterate (PHB) formed from the monomers hydroxybutyric acid. PHB is a polyhydroxyalkanoates (PHA), a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. Any physical or chemical change in polymer as a result of environmental factors, such as light, heat, moisture, chemical conditions or biological activity is termed as degradation of plastics. Photodegradation means the degradation of polymer by light. Thermal degradation means degradation of polymer by heat energy. Biodegradation is defined as any physical or chemical change in a material cause by biological activity. Microorganisms such as bacteria, fungi and actinomycetes are involved in the degradation of both natural and synthetic plastics. A number of aerobic and anaerobic microorganisms that degrade PHA, perticularly bacteria and fungi, have been isolated from various environments (Lee, 1996). Acidovorax facilis, Aspergillus fumigatus, Comamonas sp., Psedomonas lemoignei and Variovorax paradoxus are among those found in soil, while in activated sludge Alcaligenes faecalis and pseudomonas have been isolated. Comamonas testosteroni has been found in seawater. PHA degradation by Pseudomonas stutzeri in lake water has also been observed, because a microbial environment is required for degradation, PHA is not affected by moisture alone and is indefinitely stable in air (Luzier, 1992). At least two categories of enzymes are actively involved in biological degradation of polymer: Extracellular and Intracellular.

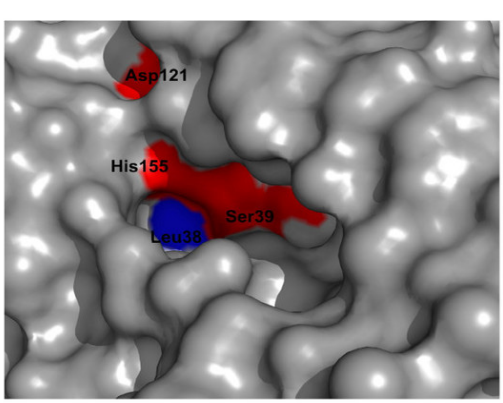

Figure 1 : Top view of the binding site of the PHB depolymerase from *Penicillium funiculosum*. The catalytic residues are marked in red, the hydrophobic residue et position x_1 of the Gx_1Sx_2G motif is marked as blue.

Analysis of their primary structures revealed that the enzymes are composed of substrate binding domain, catalytic domain and a linker, region connecting in to two domains. The substrate binding domain play a role in binding to the solid PHB. The catalytic domain contains the catalytic machinary composed of a catalytic triad (Ser-His-Asp). The serine is a part of a lipase box pentapeptide Gly-X-Ser-X-Gly, which has been found in all known hydrolases such as lipases, esterases and serine proteases (Jaeger *et al*., 1994). The properties of PHB depolymerases have been studied extensively and share several biochemical properties such as; relatively small molecular weight, below 100 kDa and most PHA depolymerases are between 40 to 50 kDa; do not bind to anion exchangers such a DEAE but have strong affinity to hydrophobic materials such as butyl-toyopearl and phenyl-toyopearl; optimum pH between 7.5 to 9.8, only the depolymerase of *Pseudomonas picketti* and *Penicillium funiculosum* have pH optima between 5.5 and 7, highly stable at a wide range of pH, temperature, ionic strength.

Material and Methods

- Materials used: A - Bushnell and Hass Minerals Medium
- Manganesium sulphate → 0.20 Gm/lit
- Calcium chloride→ 0.02 Gm/lit
- Monopotassium phosphate → 1.00 Gm/lit
- Dipotassium phosphate → 1.00 Gm/lit
- Ammonium nitrate → 1.00 Gm/lit
- Ferric chloride → 0.05 Gm/lit

Final pH is = 7.0 ± 0.2 & 3.27 gm was suspended in 1 liter of distilled water.

B. PHB as sole source of carbon

Methods

Isolation of PHB degrading oragnisams

For the isolation of PHB degrading marine organisms, the soil samples were collected from the seashore. PHB degraders were isolated by the standard liquid enrichment technique using 100 ml BHM medium in 250ml erlenmeyer flask containing 0.2% w/v PHB as sole source of carbon. These flasks were incubated on rotary shaker at 30^0 C till the growth was observed.Culture was streaked on PHB agar plate containing 3% NaCl, colony showing the zone of clearance was selected for further studies. For Identification fungal mycelia was observsd by using lactophenolblue stain.

Biodegradation studies of synthetic plastics and PHA

Studies on biodegradation of polymers (synthetic plastic like polythene & PHA) were carried out using the Bushnell Hass Minerals Medium containing polymers as a sole source of carbon. The

flasks were inoculated with fungal isolate (1.17 x 10^7 spors/ml) seperately and incubated at 30°C under shaking as well as static condition. Samples of 2 ml each were withdrawn at an alternative days over a period of a month. The samples were analyzed for extracellular protein concentration (lowry et al., 1951) and % weight loss of both polymers in each.

fungal strain maintenance & enzyme production:
The fungal isolate MA was maintained on Potato dextrose agar and used for PHB depolymerase production. PHB depolymerase production was carried out in Bushnell Hass Minerals (BHM) Medium containing PHB (0.2%) as sole carbon source at 30°C for 5 days. The culture broth was centrifuged at 8000 rpm for 20 min and the filtrate was used as source of enzyme. PHB depolymerase was assayed from the supernatant by measuring the hydrolysis of PHB granules leading to a decrease in turbidity at A_{660} nm. The PHB depolymerase assay was performed according to method described by Jendrossek et al., 1993; Kita et al., 1995. Protein estimation was carried out using Folin phenol reagent (Lowry et al., 1951).

Once all parameters were optimized further PHB depolymerase production was carried out at optimum conditions. The fermentation broth was centrifuged at 9000 rpm for 20 min to remove cell mass and supernatant was subjected to precipitation using ammonium sulfate. The precipitated enzyme was purified by gel filtration using Sephadex G-75, granule size 40-120 µm, bed volume 15-20 ml per gram.

Characterization of PHB depolymerase
SDS-PAGE was carried out in a vertical slab apparatus. Stacking gel of 5% and resolving gel of 10% acrylamide concentration contained 0.1% (w/v) SDS. The samples were pre incubated 95^0C for 4 min with sample buffer. Electrophoresis was carried out at room temperature using running buffer system (tris base, Glysine, SDS, Distilled water). Protein bands were visualized by silver staining. PMWH used as a protein molecular weight standard.

Results
Isolation of PHB degrading organisms
PHB degrading fungus was isolated on the basis of degradation which shows clear zones surrounding the colony. Culture No.5 was selected on the basis of their ability to utilized PHB.

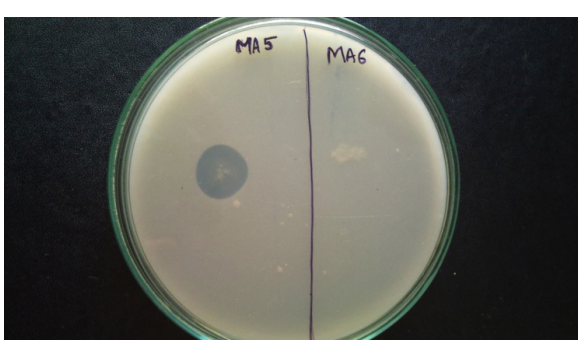

Figure 2: fungus shown zone of clearance on PHB agar plate.

% Weight loss of polymer:
Percent weight loss of polymer in each flask was calculated after a month considering the respective initial weight of polymer taken for the analysis. Higher % Weight loss of polymers was observed in case of shaking condition (12%) than static condition (7%) for MA5.

Growth and extracellular protein concentration profile

MA5 showed the higher enzyme production in shaking condition (100 µg/ml) as compared to static condition (30.07 µg/ml) in presence of LDPE. As number of days increase the enzyme production increase. The maximum enzymes were produced at 14th day then after it was decreased. Moderate amount of enzymes were produced in presence of PHA.

PHB depolymerase production by PHB degrading fungal cultures
Fungal culture MA5 (1.93 U/ml) showed maximum PHB depolymerase production at 72 h (3rd day).

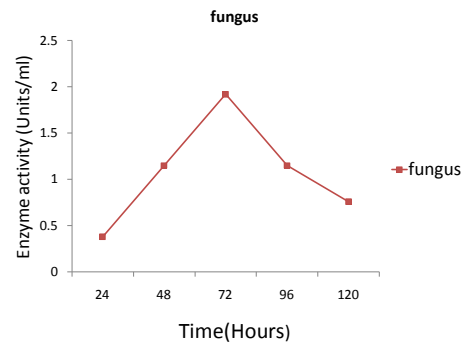

Figure 3 PHB depolymerase production of PHB degrading fungal culture.

Optimization of cultural parameters for maximum PHB depolymerase production:

Optimization of initial medium pH:
PHB depolymerase production were checked at different pH. The maximum PHB depolymerase production 3.07 U/ml was observed for fungal culture at pH 6.0 and 2.69 U/ml for bacterial culture at pH 7.0

Optimization of temperature:
When the cultures were inoculated in BHM containing PHB (0.2% w/v) and incubated at temperatures ranging from 25-42°C, maximum PHB depolymerase production of 2.69 U/ml and 1.92 U/ml was observed at 30°C for both cultures. (Figure 9(b)).

Optimization of substrate concentration:
PHB was provided at different concentration of 0.1, 0.15, 0.2, 0.25 (% w/v) for PHB depolymerase production. Initial concentration of 0.2% w/v showed maximum PHB depolymerase production at the end of 72 hr (figure 10). On increasing PHB concentration to 0.35 % PHB depolymerase production was suppressed.

Production of PHB depolymerase at optimized conditions:
fungal culture showed maximum PHB depolymerase production of 2.88 U/ml at 30°C and pH 6 with substrate concentration of 0.2%(w/v). After that we proceeded for purification of enzyme by ammonium sulfate, dialysis of enzyme and gel permeation chromatography.

Purification of PHB depolymerase
The enzyme was precipitated out with 0-100% ammonium sulfate saturation. Obtained specific activity was 40.80 U/mg with MP5. Enzyme purified by gel permeation chromatography.

Table 1: Purification of PHB depolymerase of fungal culture

Treatment	Volume of sample (ml)	Unit Activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Fold purification

Crude	100	0.60	0.084	7.14	1
Ammonium sulphate precipitation	70	2.53	0.062	40.80	5.71
Dialysis	2	4.92	0.056	87.85	12.30

Summery And Conclusion

The screening and isolation of PHB degrading microorganisms were done on the basis of the ability to produce extracellular PHB depolymerase on PHB containing BHM agar plates. Those isolates were selected which were produced clear zone on PHB agar plate due to hydrolysis of PHB. The optimal condition for PHB depolymerase production was found to be at 30 °C and pH 6 for fungal culture and the optimum enzyme production was obtained with initial substrate concentration of 0.2%(w/v) in shaking condition.

The PHB depolymerase from isolate was partially purified, fractioned and characterized. The optimum temperature for enzyme activity were at 45 ° C. The optimum pH was pH 8. The Km and Vmax values for fungus were 0.333 mg/ml and 526.31 U/min respectively. Microorganisms possess PHB degrading ability.