International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.4, No.1, pp 430-437, Jan-Mar 2012

Production of Poly (β) hydroxybutyrate (PHB) depolymerase PHAZ $_{Pen}$ from *Penicillium expansum* by Plackett-Burman design: Partial purification and characterization.

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Abstract: Production of Poly (β-) hydroxybutyrate (PHB) depolymerase of *P.expansum* was optimized by Plackett Burman design. The main effect of each variable upon PHB degradation and the production of PHB depolymerase enzyme was estimated. Within the range of the tested levels of variables, all parameters tested affected activity at low levels. pH at both acidic and alkaline levels did not show significant influence on *P.expansum* depolymerase production. Presence of glucose showed repressive effect on the enzyme activity suggesting the catabolic repression effect of glucose on PHB depolymerase production. Of all the factors tested, aeration, nitrogen, KH_2PO_4 levels and inoculum density showed profound effect on PHB depolymerase activity at the levels tested, indicating the importance of these factors in production of the enzyme. According to the data obtained from the Plackett-Burman experimental results, KH_2PO_4, and NH_4Cl should be added in the lowest concentration and the medium predicted to be near optimum should be of the following composition (g/L): PHB, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4Cl, 0.5; KH_2PO_4, 0.25; $CaCl_2$, 0.02; $FeCl_3$, 0.05. The statistical analysis of the results showed that, in the range studied, all the factors had a significant effect ($p < 0.05$) on PHB depolymerase production. *P. expansum* produced maximum PHB depolymerase (~ 6U/mL) by 48 h when grown in BHM containing 0.05 %, w/v PHB, pH 5.0 at 30°C. Under optimized culture conditions, maximum PHB depolymerase activity was achieved after 2 days as against 4 days in the un-optimized conditions with a 2 fold increase in activity. Partial purification of the extracellular (PHB) depolymerase $PHAZ_{Pen}$ from *P.expansum* by single step affinity chromatography using PHB yielded 7.36 fold purity and almost 98 % recovery of protein. The enzyme composed of a single polypeptide chain of 20 KDa, as determined by SDS- PAGE. The enzyme stained positive for glycoprotein by PAS staining.
Key words: PHB depolymerase, *P.expansum*, production, Plackett- Burman design, purification, characterization.

INTRODUCTION

Polyhydroxyalkanoates, PHAs, are versatile biopolyesters synthesized by numerous bacterial strains as intracellular storage compounds of carbon and energy. These polyesters can be divided primarily into two classes of shortchain-length (SCL) PHAs and medium-chain-length (MCL) PHAs, according to thecarbon-chain-length of constituents: SCL-PHAs consist of (*R*)-hydroxyalkanoates of C3-C5and MCL-PHAs are comprised of (*R*)-hydroxyalkanoates of C6-C14. Poly (3-hydroxybutyrate) (PHB), a representative

SCL-PHA, is a promising material for use as a renewable and biodegradable plastic. [1,2]

It has been reported that PHB-degrading bacteria are distributed widely in the natural environment, and several extracellular PHB depolymerases with differing biochemical properties have been isolated from various bacterial origins[3,4]. However, the ability to degrade SCL-PHA is not restricted to bacteria, and some filamentous fungi also play an important role in the extracellular degradation of SCL-PHAs such as PHB and its copolyesters with 3-hydroxyvalerate[5].

The used PHB is normally buried under the ground and can be decomposed by the extracellular PHB depolymerase secreted from the soil microbes. The fungal biomass in soils generally exceeds the bacterial biomass and thus it is likely that fungi may play a considerable role in degrading polyesters, just as they predominantly perform the decomposition of organic matter in the soil ecosystem. However, in contrast to bacterial polyester degradation, which has been extensively investigated, the microbiological and environmental aspects of fungal degradation of polyesters are unclear[6].

Optimizing the parameters by statistical method reduces the time and expense; otherwise if required, conventional one-factor-at-a-time approach is used. Several statistical methods are available for optimization experiments[7]. Plackett and Burman's statistical method is one of such approaches involving a two-level fractional factorial saturated design that uses only k + 1 treatment combinations to estimate the main effects of k factors independently, assuming that all interactions are negligible[8]. Saturated designs are used in the early stages of experimentation to screen out unimportant factors from among a large number of possible factors. In full factorial designs, the number of factors increases exponentially leading to an unmanageable number of experiments[9]. Hence, fractional factorial design like Plackett–Burman becomes a method of choice for initial screening of medium components.

Many reports have been published on the fungal degradation of SCL-PHAs in the environment, however reports on the properties of the PHB depolymerases from fungi are relatively rare. Only few fungal PHB depolymerases have been purified and partially characterized to date[10-16].

The present work describes the optimization of growth parameters for maximum PHB depolymerase production by Plackett- Burman design from a fungal isolate, *P.expansum*. The enzyme was also partially purified by single step affinity chromatography and characterized. To our knowledge there are only a few reports on the biochemical characteristics of fungal PHB depolymerases and no report so far on statistical optimization of medium components for production of any fungal PHB depolymerase.

MATERIALS AND METHODS

PHB

PHB was obtained as a kind gift from Biomer Inc, Germany. The molecular weight of PHB was 470,000 g/mol. All experiments were performed using PHB powder.

Isolation of a SCL-PHA-degrading fungus

A SCL-PHA-degrading filamentous fungus from a wastewater sample was isolated by pure culturing a colony with high depolymerase activity among fungi grown on a mineral salt agar medium[13] containing PHB as the sole carbon source.

Optimization of the medium using statistically based Plackett- Burman experimental design

Experimental design

The Plackett-Burman experimental design, a fractional factorial design[8,17] was used in this research to reflect the relative importance of various environmental factors on PHB degradation and PHB depolymerase activity in liquid cultures. In this experiment, eleven independent variables were used in 22 combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested (Table 1). All trials were performed in triplicate and the averages of degradation observation results were treated as the responses. The main effect of each variable was determined with the following equation:

$$Exi = (\Sigma Mi^{+} - \Sigma Mi^{-})/N$$

where Exi is the concentration effect of the tested variable, Mi^{+} and Mi^{-} are PHB depolymerase activity in trials where the independent variable (Xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. When the sign is positive, the influence of the variable upon PHB degradation is greater at a high concentration, and when negative the influence of the variable is greater at a low concentration. Using Microsoft Excel, statistical t-values for unequal paired samples were calculated for determination of variable significance.

Table 1. Variables representing components used in Packett-Burman design.

Variable	Symbol	(-) Level	(+) Level
Time (h)	X_1	48	96
pH	X_2	5	8
Temperature(°C)	X_3	30	37
Inoculum size (%,v/v)	X_4	2	5
PHB(g/L)	X_5	0. 5	2
Glucose(g/L)	X_6	0	5
Volume/flask (mL)	X_7	1:4	1:2
Nitrogen (g/L)	X_8	0.5	1.5
KH_2PO_4 (g/L)	X_9	0.25	1.75
Yeast extract (g/L)	X_{10}	0.01	0.1
Aeration (rpm)	X_{11}	Static	100

X_1–X_{11} represent different assigned variables; the sign '+' is for high concentration of variables and '-' is for low concentration of variables in gl^{-1}.

PHB depolymerase assay

The PHB depolymerase assay was carried out by adding the enzyme to 100mM sodium citrate buffer (pH 5.0) containing 300 µg of PHB at 55ºC for 1 h. The activity was arrested by adding 0.1 ml of 1 N HCl to the 3 ml enzyme substrate system. The decrease in the turbidity was measured at 600 nm using a colorimeter. One unit of PHB depolymerase is defined as the amount of enzyme required to decrease the A600nm by 1.0 per hour[10].

Purification of PHB depolymerase

The proteins from the culture supernatant of PHB grown *P.expansum* (36-40h) were bound to PHB powder (0.5g/L) for 6-8h at 8-10ºC, the PHB filtered through Whatman No.1 filter paper and the bound PHB depolymerase was eluted with 100mM sodium citrate buffer (pH 5.0) containing 0.1%(v/v) TritonX-100, kept on magnetic stirrer at room temperature (32±2°C) for 1h. The eluant was checked for PHB depolymerase activity and protein estimated from absorbance at 280_{nm}[13].

Molecular weight determination

Electrophoresis (10 %, w/v sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out to measure the molecular weight according to Laemmli (1970)[18].

Glycoprotein staining

Carbohydrate staining of glycoprotein in SDS-PAGE gel was carried out with fuchsin-sulfate after periodate oxidation[19].

RESULTS

Microorganism: Identification and characterization

PHB degrading fungal species identified as *P.expansum* (Fungal identification service, Agarkar Research Institute, Pune) based on morphotaxonomy, was used in this work.

Optimization of PHB depolymerase production by Plackett- Burman design

The independent variables examined in Plackett-Burman design and their levels are presented in **Table 1.** The design was applied with different fermentation conditions. The main effect of each variable upon PHB degradation and the production of PHB depolymerase enzyme was estimated and presented in Fig.1. Within the range of the tested levels of variables, all parameters tested affected activity at low levels (Fig.1). pH at both acidic and alkaline levels did not show significant influence on *P.expansum* depolymerase production. Presence of glucose showed repressive effect on the enzyme activity suggesting the catabolic repression effect of glucose on PHB depolymerase production. Of all the factors tested, aeration, nitrogen, KH_2PO_4 levels and inoculum density showed profound effect on PHB depolymerase activity at the levels tested, indicating the importance of these factors in production of the enzyme.

According to the data obtained from the Plackett-Burman experimental results, KH_2PO_4, and NH_4Cl should be added in the lowest concentration and the medium predicted to be near optimum should be of the

following composition (g/L): PHB, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4Cl, 0.5; KH_2PO_4, 0.25; $CaCl_2$, 0.02; $FeCl_3$, 0.05. Fungal spores were inoculated with 2% (v/v) inoculum (containing $1x10^9$ spores/mL) of 5-day-old culture in a total culture volume of 25 ml BHM and incubated at pH 5.0 and 30ºC (Table 2). The statistical analysis of the results showed that, in the range studied, all the factors had a significant effect ($p < 0.05$) on PHB depolymerase production. *P. expansum* produced maximum PHB depolymerase (~6U/mL) by 48 h when grown in BHM containing 0.05 %, w/v PHB, pH 5.0 at 30°C. Under optimized culture conditions, maximum PHB depolymerase activity was achieved after 2 days as against 4 days in the un-optimized conditions with a 2 fold increase in activity.

Partial purification and characterization

Partial purification of the extracellular Poly (-β-hydroxybutyrate) (PHB) depolymerase $PHAZ_{Pen}$ from *P.expansum* by one step affinity chromatography using PHB yielded 7.36 fold purity and almost 98% recovery (Table 3). The enzyme composed of a single polypeptide chain of 20 KDa, as determined by SDS-PAGE (Fig.2). The enzyme stained positive for glycoprotein by PAS staining (Fig.2, lane 4).

Fig.1. Elucidation of fermentation factors affecting PHB depolymerase activity produced by *P.expansum.*

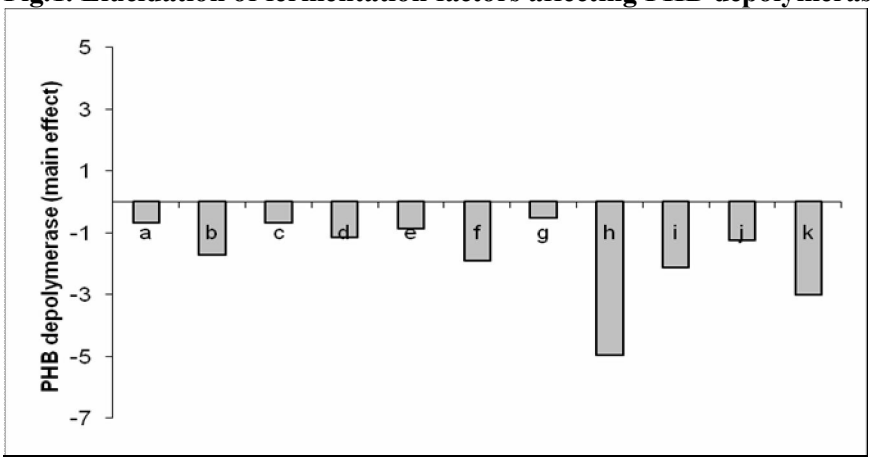


Table 2. Degree of positive or negative effect of independent variables on *P.expansum* PHB depolymerase according to the levels in the Plackett-Burman experimental design:

Variable	Symbol	(-) Level	(+) Level	T test	Main effect	Significance Level
Time (h)	a	48	96	3.33	*-0.67*	**S**
pH	b	5	8	1.50	-1.73	NS
Temperature(°C)	c	30	37	3.29	*-0.67*	**S**
Inoculum size (%,v/v)	d	2	5	16.14	*-1.14*	**S**
PHB(g/L)	e	0. 5	2	9.45	*-0.87*	**S**
Glucose(g/L)	f	0	5	7.56	*-1.90*	**S**
Volume/flask (mL)	g	1:4	1:2	7.01	*-0.53*	**S**
Nitrogen (g/L)	h	0.5	1.5	38.44	*-4.96*	**S**
KH_2PO_4 (g/L)	i	0.25	1.75	12.67	*-2.13*	**S**
Yeast extract (g/L)	j	0.01	0.1	9.1	*-1.23*	**S**
Aeration (rpm)	k	static	100	52.63	*-3.0*	**S**

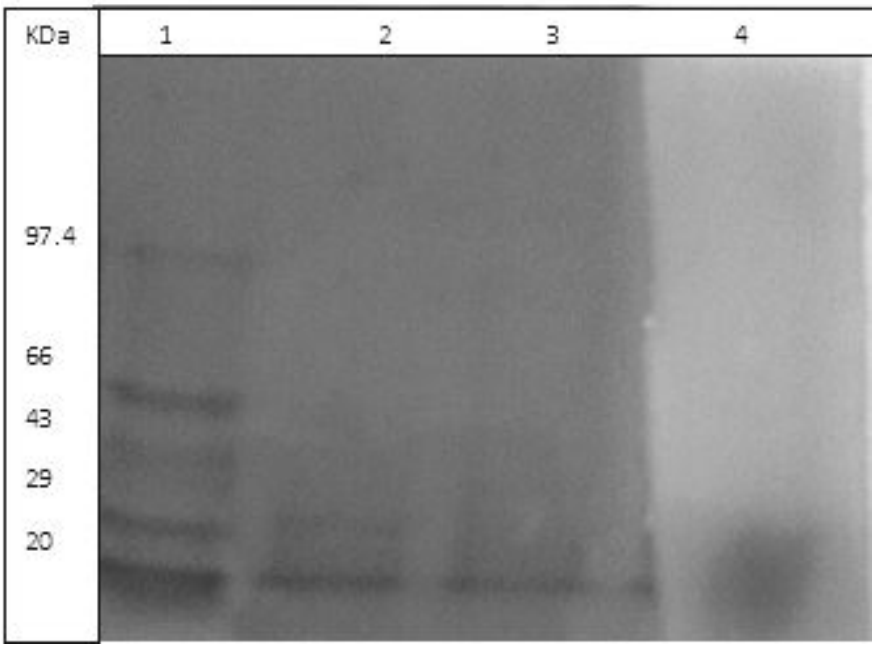
Bold type indicates positive effect, italic type indicates negative effect.
Significant (S); Non-Significant (N.S). $t_{0.05}$= 2.92

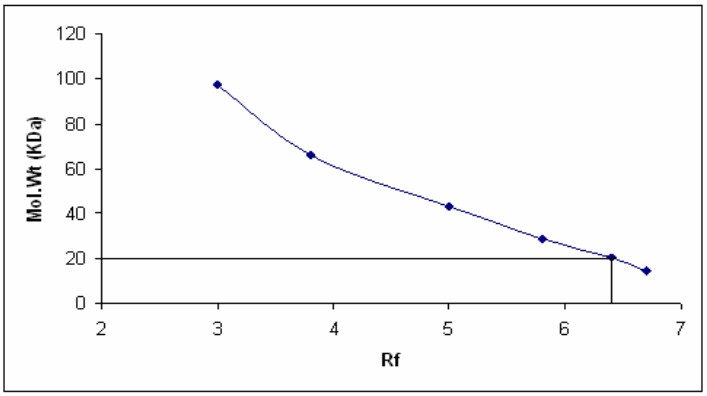
Table 3. Purification of *P.expansum* PHB depolymerase.

Sample	Activity (U/mL)	Total activity (Units)	Protein (µg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% Recovery
Crude (25)	6.84	6840	80	80	85	1	100
Affinity chromatography using PHB (25mL)	67	167.5	28.7	0.718	233	2.74	98

Fig.2a. SDS denatured PAGE- Standard molecular weight marker [Coomassie stained (Lane 1), Purified PHB depolymerase by Affinity Chromatography using PHB (Lane 2 & 3), Glycoprotein staining of the purified PHB depolymerase by PAS method (Lane 4).

b. Molecular weight calibration by SDS-PAGE.





DISCUSSION

In order to optimize enzyme production, the Plackett-Burman design was applied, which has been demonstrated as an efficient approach to screen for medium components and/or factors affecting PHB degradation. Our results are in agreement to all other known PHB bacterial depolymerases, which are repressed in the presence of a soluble carbon source. In accordance with our results are those reported for *Pseudomonas lemoignei,* [20,21] which produced polymerase maximally during growth on succinate. This result is in good agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon in the liquid medium. Similarly, production of the PHB depolymerase from *E. minima* W2 (Pha*ZEmi*) was also significantly repressed in the presence of glucose[14]. In contrast to this observation, the PHB depolymerase of *Nocardiopsis aegyptia* was found to be constitutive[22].

Production of enzyme depends on the growth period of microorganisms[10,12]. For example, *P. Funiculosum*[15] cultured at 30°C produces the most PHB depolymerase in the stationary phase. *P.simplicissimum* grew most rapidly at 37°C. In the early stage of the growth, the enzyme production was the highest at 30°C. However, in the later part of the culture period, the enzyme production was maximal at 25°C. The enzyme production increased with the culture time and then levelled off at the stationary phase[23]. PHB depolymerases from different systems have been purified using different multi-step methods. According to the literature, the yield of PHB depolymerase after purification was 66% from *P. funiculosm*; 27% from *A. faecalis* ; 42% from *Pseudomonas* sp. ; 66% from *A. fumigatus* ; 73.5% from *Aureobacterium saperdae*; 61% from *P.simplicissimum*; 86.11% from *A.fumigatus* Pdf1 and 21.53% from *P.citrinum* S2 . The increases of the enzyme activity were 4.5 fold for *P. funiculosum*; 1.5 fold for *A. faecalis* ; and 5.67 fold for *Pseudomonas* sp. ; 2.4 fold in *P.simplicissimum* ; 33.56 fold in *A.fumigatus* Pdf1 and 16.18 fold for *P.citrinum* S2 [11-16].

The molecular weight of *P.expansum* PHB depolymerase determined here is in agreement to that of the PHB depolymerase obtained from *P. funiculosum*; *P. Pinophilum*; *P.simplicissimum* and *A.fumigatus* Pdf1 and *P.citrinum*[12,13,15,16,24] bacterial PHB depolymerase [20,25,26,27] all of which showed a single polypeptide of different molecular weights. The PHB depolymerase of *A.faecalis* AE122 and *P.citrinum* S2 are the only depolymerases reported with unusually high apparent M_r of 96 Kda and 240 KDa[16,27]. The enzyme stained positive for glycoprotein by PAS staining. Carbohydrates were not detected in the PHB depolymerase either from *A. fumigatus* or from *A.saperdae* [10,26]. However, the bacterial PHB depolymerase from *Pseudomonas lemoignei*[20]; and fungal PHB depolymerase from *P. funiculosum* [12] Brucato; *P.simplicissimum* [15]; *A.fumigatus* Pdf1[13] and *P.citrinum* S2 [16] were glycosylated. The composition and function of the carbohydrate moiety of the glycosylated PHB depolymerase and other proteins studied so far are unknown. The cellulases of *Trichoderma reesii* and *Cellulomonas fimi* are known to be glycosylated in the proline/ threonine rich region and an extracellular glucoamylase from *Aspergillus niger* is also glycosylated in the C-terminal region of the protein. A functional role of the carbohydrate moiety in binding the substrate of cellulases has been proposed. However, glycosylation is not essential for activity but may prove the resistance of the exoenzyme to elevated temperature and/or hydrolytic cleavage by proteases of competing microorganisms[28].

CONCLUSION

Biodegradable plastics, such as PHB, continue to make progress in both the commercial and scientific fields. However, their use as a replacement for conventional plastics in a wide range of applications has been hindered by their brittleness, low mechanical strength and high production cost. Improvements in the fermentation technology and genetically modified strains are being developed for the economical and efficient production of PHB [23,29]. Many fine chemicals produced by chemical processes can also be prepared using microbial fermentation[30]. A pure monomer of PHB, D-3-hydroxybutyric acid, is also an important precursor of 4-acetoxyazetidinone, which is used in making carbapenem antibiotics [31, 32]. Intensive studies on this newfound strain as a useful microorganism for industrial application are necessary.

ACKNOWLEDGEMENT

The authors wish to express their gratitude for the support extended by the management of Jain University, Bangalore, India in carrying out the research work in the Department of Microbiology.

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