International Journal Of

Recent Scientific Research

ISSN: 0976-3031
Volume: 7(3) March -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 3, pp. 9713-9717, March, 2016

International Journal of Recent Scientific Research

RESEARCH ARTICLE

DEPOLYMERASE AND ESTERASE ASSAY IN *ALCALIGENS FAECALIS* AGAINST THE COPOLYMERIZED *ACINETOBACTER JUNII* CN1 PHBV

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ARTICLE INFO

Article History:

Received December, 2015
Received in revised form 21st January, 2016
Accepted 06th February, 2016
Published online 28th March, 2016

Keywords:

Acinetobacter junii CN1 PHBV, *Alcaligens faecalis*, Depolymerase / esterase Assay.

ABSTRACT

With a view to improve the mechanical properties of *Acinetobacter junii* CN1 PHB, it was copolymerized with commercial PHV. The copolymerized PHBV when subjected to soil burial assay a total of 6 PHBV degrading bacterial strains (S1- S6) were isolated not only from the soil in which the PHBV was buried but also from the surface of the degraded PHBV membrane. Further, the PHBV degrading bacterial strains of the present investigation were identified as *Bacillus*, *Bifidobacterium*1, *Bifidobacterium* 2, *Corneybacterium*, *Bifidobacterium* 3 and *Alcaligenes faecalis* respectively. On screening the six bacterial isolates, the maximum zone of clearance was reported with *Alcaligens faecalis* which also showed the highest level of activity for both the esterase (0.27 U/ml) and depolymerase (0.28 U/ml). While determining the degradation of PHBV at different temperatures (30, 37, 45, 50 & 55 °C), the results revealed the better degradation of PHBV at 30 °C and 50 °C for esterase and depolymerase respectively. However, the pH optima for both the PHBV depolymerase and esterase of *A.faecalis* were found to be 7 and 8 respectively.

INTRODUCTION

Intracellular PHB, being a storage material has been produced by a variety of bacteria in response to nutritional stress (Jendrossek & Handrick, 2002; Rehm, 2003). It is normally accumulated along with some PHB binding proteins and existing in an amorphous state. Subsequently, PHB will be degraded by several hydrolases. The degradative products would be then utilized by the organism itself as a source of carbon and energy (Thompson *et al.*, 1994). In general, the intracellular PHB depolymerases can function at the late stationary phase *of Bacillus* Sp. It has been first identified in *Rhodospirillum rubrum* with two components *viz*., a thermo stable activator and thermo labile esterase (Merrick & Doudoro, 1964; Handrick *et al.*, 2004).

Further, the process of degradation of biopolymer in the environment is being mediated through the microbial enzymes *viz*., esterase and depolymerase (Delafield *et al*., 1965; Jendrossek *et al*., 1996; Jendrossek, 2001). These extracellular enzymes break down the complex polymer into soluble products of short chains / smaller molecules *viz*., oligomers, dimmers and monomers. The resulting short chain length molecules are being further mineralized into the end products such as CO_2, methane and H_2O. Which in turn will be further utilized as the carbon and energy sources (Gu, 2003; Sathianachiyar & Aruna, 2013) by the other soil microbes.

Similarity between intracellular PHB depolymerase of *R.rubrum* with extracellular PHB depolymerases of *Acidovoras* Sp. has been reported by Kobayashi *et al.* (1999). Further, right from the onset of development of biodegradable plastics, it is believed that they can be potentially cleaved by microbial enzymes due to their hydrolysable ester bonds. Yamamoto – Tamura *et al.* (2015) have measured the soil esterase activity by using pNP valerate as a substrate. Esterase activity for p-nitrophenyl acetate (pNP acetate) in soil can be used as an indicator of polybutylene succinate degradation (Sakai *et al*., 2003). Very recently, PHB with improved mechanical properties on copolymerization with other polymers, finds its wide applications in every sphere of life (Bucci & Tawares, 2005; Chen & Wu, 2005; Park *et al*., 2005; Noda, 2001; Zinn *et al*., 2001). Therefore, the present study attempts to evaluate the depolymerase and esterase assay for the PHBV degrading microorganisms of the environmental samples experimented for soil burial assay.

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MATERIALS AND METHODS

Copolymerization of Acinetobacter junii CN1 PHB

The PHB extracted from *Acinetobacter junii* CN1 (S.Poornima, Ph.D., Thesis. Madurai Kamaraj University) was copolymerized with commercial polyhydroxy valeric acid (Sigma) (1:1 ratio) by dissolving in 5 ml chloroform and fabricated into a film by casting in clean, dry, glass Petri dish in a fume hood (Woolnough *et al*., 2008)**.** The film was further dried for 24 hrs. The dry copolymerized PHBV film was then removed from the Petri dish using a razor blade and subjected to physical and molecular characterizations.

Isolation of copolymerized PHBV degrading microorganisms

Garden soil sample used in biodegradation assay was serially diluted. From 10^{-6} dilution, 0.1 ml was taken and inoculated in a sterilized plate with mineral salt medium containing PHBV which was fabricated with the extracted PHB obtained on sonication (Sonication for 2 hrs at 90 duty cycles) and commercial PHV (glucose substitute) in 1: 1 ratio. The plates were then incubated at 37 °C for 72 hrs. The isolates which produced the maximum zone of clearance (Augusta, *et al*., 1993) were then subjected not only to morphological and biochemical characterizations (Cappuccino and Sherman, 2013) but also to molecular characterization by 16S rRNA sequencing (S.Poornima, Ph.D., Thesis. Madurai Kamaraj University).

Esterase assay

Esterase activity of the bacterial strain was determined by adopting the method proposed by Eggert *et al*. (2000). Accordingly, the ρ-nitrophenyl ester of acetate (0.8 mM) was dissolved in 10 ml isopropanol and mixed with 90 ml sodium phosphate buffer (pH 8) supplemented with sodium deoxycholic acid (207 mg) and gum Arabic (100 mg). The final concentration of the substrate was 0.8 mM. The culture supernatant dissolved in10 µl 2 mM glycine, (pH 11) was added to 2.5 ml substrate emulsion (copolymer) after a brief spell of 10 min at 37 °C. The absorbance was recorded at 410 nm.

Effect of temperature

To determine the effect of temperature on the growth of bacterial consortium and production of esterase, the experiment was executed at 30, 37, 40, 45 and 50 °C, at 150 rpm and pH 7.0.

Effect of pH

Similarly, the Effect of pH on the growth and production of esterase was assayed at pH 5.0, 6.0, 7.0, 8.0 and 9.0 at 37 °C and 150 rpm.

Depolymerase assay

Activity of depolymerase was assayed according to the method described by Kobayashi *et al*. (1999). Accordingly, 0.1 % PHB or 0.3% PHV was added to 50 Mm Tris- HCl buffer (pH 7-8) against the bacteria. The suspension of copolymer was incubated for 20 min in 300 ml flask immersed in an ultrasonic water bath. Culture supernatant (0.1 ml) was then added to 0.9 ml substrate suspension and incubated for 24 hrs at 30 °C. Depolymerase activity was then measured in terms of turbidity of PHB and PHV suspension at 650 nm.

Effect of temperature and pH

The optimal levels of the physicochemical variables *viz*., temperature (30, 37, 40, 45 & 50 °C) and pH (5.0, 6.0, 7.0, 8.0 & 9.0) were also determined for their effect on the growth of bacterial consortium and the production of the enzyme depolymerase.

RESULTS AND DISCUSSION

The copolymerized PHBV (Plate 1) when subjected to soil burial assay (Plate 2), a total of 6 PHBV degrading bacterial strains (S1- S6) were isolated not only from the soil in which the PHBV was buried but also from the surface of the degraded PHBV membrane. The isolates were then identified based on their morphological and biochemical characteristics.

Plate 1 Copolymerized PHBV

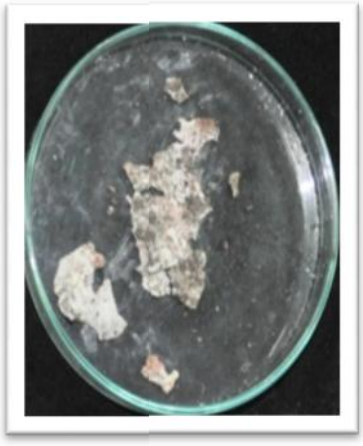

Plate 2 PHBV subjected to soil burial Assay

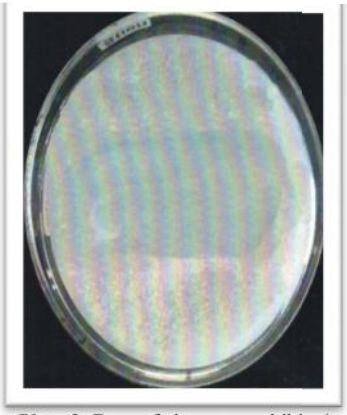

Plate 3 Zone of clearance exhibited by PHBV degrading *A.faecalis*

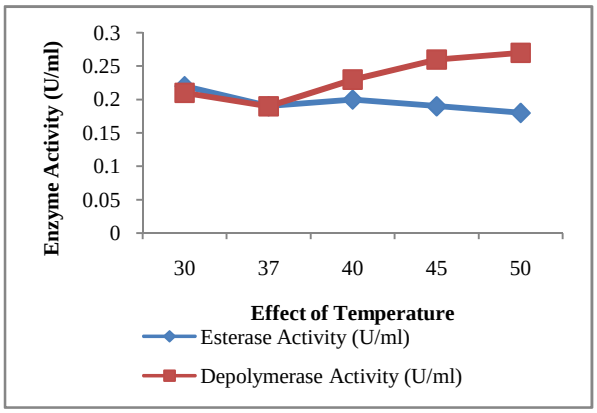

Figure 1 Effect of temperature

With the results (Table 1) of the morphological and biochemical characterizations, the PHBV degrading bacterial strains S1, S2, S3, S4, S5 and S6 of the present investigation were identified as *Bacillus*, *Bifidobacterium*1, *Bifidobacterium* 2, *Corneybacterium*, *Bifidobacterium* 3 and *Alcaligenes faecalis* respectively.

Further, on screening the six bacterial isolates of the present study, for their PHBV degrading potential, the maximum zone of clearance was reported with *A. faecalis*

Most of the PHB degrading microorganisms consist of a wide range of activity at ambient or mesophilic temperatures. While only a few species of *Bacillus* strain TT96 (Tansengco & Tokiwa, 1998) and *Streptomyces* strain MG (Tokiwa & Calabia, 2004) are reported to have the capablility of degradation at higher temperatures. Still there is little information on microbial degradation of PHB at high temperatures. In the present study, degradation of PHBV was determined at different temperatures (30, 37, 45, 50 & 55 °C) for both the enzymes (esterase & depolymerase).

Table 1 Morphological and biochemical characteristics of PHBV degrading bacteria

Sl. No.	Characterization	Interference					
	Sample	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
1.	Gram reaction	+	+	+	+	+	+
2.	Shape	Rod	Rod	Rod	Rod	Rod	Rod
3.	Motility	Non motile	Non motile	Non motile	Non motile	Non motile	Non motile
4.	Colony morphology	White irregular colonies	Light white, smooth, flat, irregular colonies	Light cream, smooth, flat, irregular colonies	Dull white small, flat, circular colonies	Light cream, smooth, flat, irregular colonies	Light cream, smooth, flat, irregular colonies
5.	Indole test	-	-	-	-	-	-
6.	Methyl red test	+	+	+	+	+	+
7.	Voges- proskauer test	+	-	-	-	-	-
8.	Citrate test	+	-	-	+	-	-
9.	Catalase test	-	-	-	+	-	-
10.	Oxidase test	+	-	-	-	-	-
11.	Triple- sugar iron agar test	+	+	+	+	+	+
12.	Urease test	+	+	+	+	+	+
13.	Gelatin hydrolysis test	+	+	+	+	-	+
14.	Probable identification	*Bacillus*	*Bifido Bacterium* 1	*Bifido Bacterium* 2	*Corney Bacterium*	*Bifido Bacterium* 3	*Alcaligenes faecalis*

(Plate3). This is mainly due to the hydrolysis of the suspended polyesters by the target organism. Besides, among the 6 organisms evaluated for enzyme assay, *A. faecalis* showed the highest level of activity for both the esterase (0.27 U/ml) and depolymerase (0.28 U/ml).

Kodama *et al*. (2009) have opined that some soil microorganisms do produce a wide variety of enzymes having esterase activity with specific substrate preferences. For example, cutinase of *Fusarium solani* prefers pNP - butyrate to pNP - acetate as substrate and cutinase like enzyme of *Cryptococcus* Sp S-2 which prefers pNP caproate to pNP butyrate and pNP- acetate. Thus the PHB degrading microorganisms with special reference to *A. faecalis*, optimum temperature, pH and other conditions were expected to be varied.

The results revealed the better degradation of PHBV at 30 °C and 50 °C for esterase and depolymerase respectively (Figure 1 & 2; Table 3). On contrary, Takaku *et al*. (2006) have reported with optimal activity for extracellular depolymerase at 65° C.

Table 3 Effect of temperature on enzyme activity

Enzyme Activity(U/ml)					
Effect of Temperature			**Effect of pH**		
Temperature	**Esterase**	**Depolymerase**	**pH**	**Esterase**	**Depolymerase**
30	0.22	0.21	4	0.21	0.149
37	0.19	0.19	5	0.19	0.154
40	0.20	0.23	6	0.23	0.159
45	0.19	0.26	7	0.26	0.167
50	0.18	0.27	8	0.24	0.189

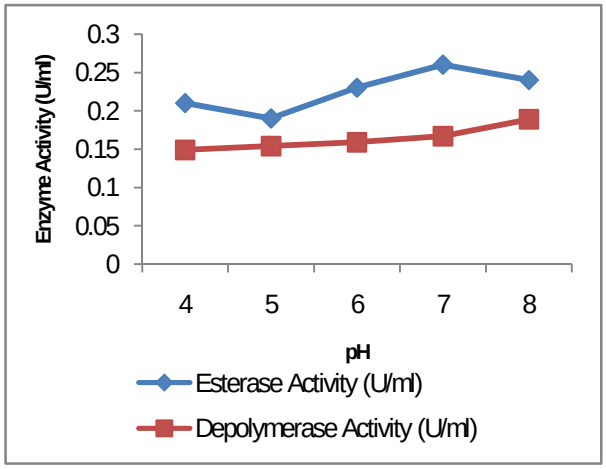

Figure 2 Effect of pH on enzyme activity

Further, the pH optima for PHBV depolymerase and esterases were found to be 7 and 8 respectively for *A.faecalis* of the present study. Whereas, many fungi show broad pH optima from 5.5 to 7.5, (Ellaiah *et al*., 2004). The maximum activity of extracellular PHB depolymerase produced by *B. megaterium* N-18-25-9, has been observed at pH 9.0 (Takaku *et al*., 2006). Briese *et al*. (1994) while exploiting sewage sludge as inoculum, they have reported with the highest degradation rates between the pH 7.5 and pH 8.0. Qin *et al*. (2006) for their turn have reported the optimal activity of PHB depolymerase of UV mediated mutagenic *Penicillium* Spp., DS9713a01 at pH 8.6.

CONCLUDING REMARKS

Identification and exploitation of potential PHBV degrading organisms and standardization of pH and temperature optima for the degradative enzymes such as depolymerase and esterase would accelerate the process of degradation of PHBV. Thereby contributes to the solid waste management.

Acknowledgement

The Authors gratefully acknowledge the Ministry of Environment and Forestry, Govt. of India, New Delhi for the financial assistance and the Management SFR College for Women for having extended the infrastructure.

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How to cite this article:

Poornima S and Kasthuri J.2016, Depolymerase and Esterase Assay In *Alcaligens Faecalis* Against The Copolymerized *Acinetobacter Junii* Cn1 Phbv. *Int J Recent Sci Res.* 7(3), pp. 9713-9717.

TSSN 0976-3031