บทความวิจัย

สภาวะที่เหมาะสมต่อการผลิตเอนไซม์ย่อยสลายอะลิฟาติกพอลิเอสเทอร์จากเชื้อ *Actinomadura* sp. strain TF1

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บทคัดย่อ

แอคติโนมัยสีทชอบร้อน *Actinomadura* sp. สายพันธุ์ TF1 แยกจากดินคอมโพสต์ในประเทศไทยและมีความสามารถในการย่อยสลายพลาสติกชีวภาพ ได้แก่ พอลิแลกติกแอซิด (PLA) พอลิแอล-แลกติกแอซิด (PLLA) พอลิคาโปรแลคโตน (PCL) พอลิบิวทิลีนซัคซิเนต (PBS) พอลิบิวทิลีนซัคซิเนต-โค-อะดิเพท (PBSA) และ พอลิไฮดรอกซีบิวทิเรต (PHB) เมื่อเพาะเลี้ยงบนจานอาหารแข็งพอลิเอสเทอร์ ที่อุณหภูมิ 45 องศาเซลเซียสเพื่อศึกษาลักษณะทางฟีโนไทป์และการวิเคราะห์ด้วยยีน 16S rDNA พบว่ามีความเหมือนกับเชื้อ *Actinomadura miaoliensis* (ความคล้ายคลึง 99 เปอร์เซ็นต์) PBS ถูกเลือกเป็นสับสเตรทเพื่อทดสอบการทำงานของเอนไซม์ PBS depolymerase จากสายพันธุ์ TF1 ทั้งรูปแบบการย่อยสลายในแผ่นฟิล์ม PBS (ความหนา 100 ไมโครเมตร) และแบบอิมัลซิฟายเออร์ PBS ในอาหารเหลว basal medium ผลการทดลองพบว่าแผ่นฟิล์ม PBS มีน้ำหนักแผ่นลดลง 60 เปอร์เซ็นต์ หลังจากบ่มเป็นระยะเวลา 90 วัน และเมื่อศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดของแผ่นฟิล์มที่บ่มไว้เป็นระยะเวลา 30 วัน แสดงถึงการเปลี่ยนแปลงโครงสร้างบริเวณผิวหน้าฟิล์ม เช่น เกิดการกร่อนและเกิดรูเมื่อเปรียบเทียบกับแผ่นฟิล์ม PBS ที่ไม่ได้เติมเชื้อ การทำงานสูงสุดของเอนไซม์ PBS depolymerase เท่ากับ 21.8 ± 1.96 ยูนิตต่อมิลลิลิตร อุณหภูมิและค่าความเป็นกรด-เบสที่เหมาะสมต่อการทำงานของเอนไซม์คือ 45 องศาเซลเซียส และ 7.0 ตามลำดับเมื่อใช้ PBS เป็นแหล่งคาร์บอนและสารสกัดจากยีสต์ร่วมกับแอมโมเนียมซัลเฟตเป็นแหล่งไนโตรเจนสำหรับการเลี้ยงเชื้อ

คำสำคัญ: *Actinomadura* sp. เอนไซม์ย่อยสลายพอลิเอสเทอร์ ไบโอพลาสติก พอลิบิวทิลีนซัคซิเนต พอลิบิวทิลีนซัคซิเนต-โค-อะดิเพท พอลิไฮดรอกซีบิวทิเรต พอลิแลกติกแอซิด

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Optimization for Production of Aliphatic Polyester-Degrading Enzyme From *Actinomadura* sp. Strain TF1

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ABSTRACT

A thermophilic actinomycete, *Actinomadura* sp. strain TF1 was isolated from compost soil in Thailand and had the ability to degrade the biodegradable plastics such as poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(ε-caprolactone) (PCL), poly (butylenes succinate) (PBS), poly(butylenes succinate-*co*-adipate) (PBSA) and poly(β-hydroxybutyrate) (PHB) when cultured on polyester agar plates at 45°C. According to phenotypic characteristics and 16S rDNA gene analysis was identified as *Actinomadura miaoliensis* (99% similarity). PBS was selected as substrate to demonstrate PBS depolymerase enzyme activity by strain TF1 both PBS film (thick 100 μm) and emulsified PBS in basal medium. The results showed that PBS film lost weight of 60% after 90 days and the scanning electron microscopy of film at 30 days of incubation showed many changes in surface structure, such as erosion and pit formation as compared to the untreated PBS film. The highest activity of PBS depolymerase was 21.8±1.96 U/ml. The optimum temperature and pH were 45°C and 7.0, respectively when using PBS as carbon source and yeast extract plus ammonium sulphate as nitrogen source for cultivation.

Keywords: *Actinomadura* sp., polyester-degrading enzyme, bioplastic, poly(butylene succinate), poly(butylene succinate-*co*-adipate), poly(hydroxybutyrate), poly(lactic acid)

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Introduction

A wide variety of synthetic plastics are produced and gradually increased for uses in various parts of the world which cause the accumulation of plastics waste in the environments. However, researchers try to find new products that can be completely degraded in natural ecosystems [1]. In recent years, some types of aliphatic polyesters were developed as biodegradable plastics such as poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(ε-caprolactone) (PCL), poly(butylenes succinate) (PBS) poly(butylenes succinate-*co*-adipate) (PBSA) and poly(β-hydroxybutyrate) (PHB) which can be degraded into water and CO_2 by microorganisms [2].

To date several aliphatic polyester-degrading thermophilic actinomycetes and enzyme that can degrade bioplastics have been reported e.g. *Amycolatopsis* sp. strain K104-1 [3], *Actinomadura miaoliensis* strain BC44T-5^T [4], *Actinomadura keratinilytica* strain T16-1 [5], *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2 [6], *Thermomonospora fusca* [7] and *Laceyella sacchari* LP175 [8]. Many different types of enzyme are found in bioplastics-degrading microorganisms, such as esterase from *Bacillus smithii* strain PL21 [9], lipases from *Acidovorax* sp. strain BS-3 [10], *Burkholderia cepacia* PBSA-1 [11], *Pseudomonas aeruginosa* PBSA-2 [11], cutinase from *Fusarium solani* [12], *Pseudozyma antarctica* JCM 10317 [13] and *Aspergillus oryzae* [14], thermoalkanophilic esterase from *Streptomyces* sp. strain IN1 [15], protease from *Laceyella sacchari* LP175 [8], serine protease from *Actinomadura keratinilytica* strain T16-1 [5] and chitinase from *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2 [6].

PBS is a kind of commercially used biodegradable aliphatic polyester with a range of interesting properties, such as good mechanical properties, melts processing, biodegradability and compostability [16]. PBS is synthesized by the condensation reaction of succinic acid, butanediol, aliphatic dicarboxylic acid and alkyldiols [17]. A few reports have investigated the biodegradability of PBS and PBS-degrading enzymes produced by microorganisms. Uchida *et al* [10] cloned PBS depolymerase *(pbsA)* from *Acidovorax delafieldii* strain BS-3 and was considered to be a kind of lipase that was found to have similarities with lipase of *Streptomyces* sp. and *Mollaxella* sp. The biodegradability of PBS was slower than that of PBSA in compost soil [18]. In this study, we isolated thermophilic actinomycete which exhibited the ability to degrade both PBS film and emulsified PBS in basal medium and optimized PBS depolymerase for improving enzyme production.

Meterials and Methods

Substrate and chemicals

Poly(butylene succinate) (PBS), polyhydroxybutyrate (PHB), poly(lactic acid) (PLA) with molecular weight of 5,700 and polycaprolactone (PCL) with molecular weight of 70,000 were obtained from Sigma-Aldrich Chemical (USA). Poly (L-lactic acid) (PLLA) and poly(butylene succinate-*co*-adipate) (PBSA) were supplied from Prof. Dr. Fusako Kawai at Kyoto Institute of Technology (KIT), Kyoto, Japan. PBS film was kindly provided by Assistant Prof. Dr. Nawadon Petchwattana from Srinakharinwirot University, Bangkok, Thailand. All other chemicals used were of the highest grade available.

Screening and isolation of polyester-degrading bacteria

Polyester-degrading bacteria were isolated from composts soils in Thailand by the ten-fold serial dilution and spread plate method using polyester agar plate and incubated at 45°C for 10 days. Polyester agar plate was prepared as follows: 1 g of each polyester pellet was dissolved in 20 ml dichloromethane. The solution was emulsified with ultrasonicator into one liter of basal medium. One liter of the basal medium was composed of 200 mg yeast extract, 1 g $(NH_4)_2SO_4$, 200 mg $MgSO_4.7H_2O$, 100 mg NaCl, 20 mg $CaCl_2.2H_2O$, 10 mg $FeSO_4.7H_2O$, 0.5 mg $Na_2MoO_4.2H_2O$, 0.5 mg $Na_2WO_4.2H_2O$, 0.5 mg $MnSO_4$, 1.6 g K_2HPO_4, 200 mg KH_2PO_4 and 20 g agar at pH 7 as the modified method described by Nishida and Tokiwa [19]. The dichloromethane was removed by incubation at 50°C for 30 min. The colonies forming clear zones were selected as polyester-degrading strains.

Identification of bacterial strain

Isolates were identified by 16S rDNA sequence analysis and morphological characterization. This strain was grown on yeast-malt extract agar International Streptomyces Project (ISP-2), oatmeal agar (ISP-3), inorganic salt-starch agar (ISP-4) and glycerol-asparagine agar (ISP-5) as the media of International Streptomyces Project (ISP) described by Shirling and Gottlieb [20] for growth determination, pigmentation and color of colony. Physiological characterization tests such as growth temperatures (30-60°C) and salt tolerant (NaCl final concentration from 2 to 10% w/v) were assessed using ISP-2 medium. Carbon utilization medium (ISP-9) modified from Pridham and Gottlieb medium [21] was used to investigate ability of the strain to use different carbon sources. Each carbon source was added to ISP-9 medium to give 1% (w/v) final concentration. The strains were examined for the decomposition of 1% (w/v) of xylan, avicel, carboxymethylcellulose (CMC), tributyrin and skim milk using actinomyces agar containing (1 liter) 1.5 g KH_2PO_4, 2 g K_2HPO_4, 1.4 g $(NH_4)_2SO_4$, 2 g yeast

extract, 1 g peptone, 2 ml Tween 80, 20 g agar and 1 ml of trace element solution composed of 140 mg/l of $ZnSO_4.7H_2O$, 160 mg/l of $MnSO_4.5H_2O$, 500 mg/l of $FeSO_4.7H_2O$ and 200 mg/l of $CoCl_2.6H_2O$ in distilled water. The pH was adjusted to 6.0 and supplemented with substrates as described by Techapun *et al.* [22].

Degradation of PBS films by strain TF1 and scanning electron microscopy (SEM)

The isolate was grown in 250-ml Erlenmeyer flask containing 100 ml of basal medium and 0.35 g of PBS film pieces (2 cm ×. 2 cm) of 100 μm thick. The films were sterilized by soaking in ethanol for 1 min. The flask was incubated at 45°C at 130 rpm. The flask containing basal medium and PBS film was used as a control. After incubation 30, 60 and 90 days films were recovered, washed with distilled water and dried to constant weight. The weight loss of each film was calculated by subtracting the weight after degradation from the initial weight of the film. The surface topology of the PBS film pieces was analyzed through scanning electron microscopy (SEM) to check the structural changes after incubation and compared with PBS film in control flask.

Assay of PBS depolymerase

Enzyme activity was measured by a minor modified method from Oda et al. [23]. A reaction mixture composed of 0.5 ml of crude enzyme supernatant plus 2.5 ml of 0.1% PBS emulsion in 100 mMTris-HCl buffer (pH 7) using an ultrasonic processor was used as substrate and then incubated at 45°C for 60 min. The turbidity derived from insoluble plastics was measured at 650 nm after 60 min. One unit (U) of PBS-degrading activity was defined as 0.1 turbidity decrease in optical density at 650 nm under the assay conditions described.

Characterization of PBS depolymerase activity

Strain TF1 was inoculated in 100 ml PBS emulsion basal medium with 0.1% (w/v) final concentration (OD_{650} = 1.2) and incubated at 45°C for 7 days on a rotary shaker (180 rpm) which checked PBS depolymerase activity every day. The culture medium was centrifuged at 12,000 rpm for 10 min to obtain the crude enzyme extract and used for analyzing enzyme activity. Effect of pH on enzyme activity, the crude enzyme extracts were assayed with different pH ranges of 7.0, 8.0 and 9.0 of 100 mMTris-HCl buffer for different incubation time. Effect of temperatures was carried out at 30, 40, 45, 55 and 60°C with 100 mM Tris-HCl buffer for pH 7.0.

Optimization of culture condition for PBS depolymerase production

Different cultures conditions for maximum production of PBS depolymerase enzymes were optimized and investigated at different carbon source and nitrogen source in basal medium. PBS emulsion basal medium with 0.1% (w/v) final concentration in 100 mMTris-HCl buffer (pH 7.0) was used as substrate for enzyme activity determination. All the experiments were performed in a 250 ml flask that has 20 ml of basal medium. The inoculated medium was incubated with rotary shaking at 150 rpm for 4 days. The culture medium was centrifuged at 12,000 rpm for 10 min to obtain the crude enzyme extract and used for analyzing enzyme activity. Effect of carbon sources, enzyme production was observed at various carbon sources of 0.1% (w/v) sucrose, glucose, fructose, PLA, PBS and PBSA. Effect of nitrogen sources, various nitrogen sources of 0.12% (w/v) such as yeast extract, ammonium sulphate, casein, gelatin and yeast extract with ammonium sulphate were examined.

Results and Discussion

Isolation and identification of polyester-degrading strains

Total sixty actinomycetes strains were collected from compost soils in Thailand. After incubation for 4 days at 45°C, the colonies were appeared on polyester agar plates. Strain TF1 could degrade all of bioplastics and had the highest clear zone on PBS-agar plate then was selected as the best strain to study. The result suggested that strain TF1 had a wide range of degradation activities of biodegradable plastics. A circular clear zone formed surrounding each colony as shown in Figure 1. This happens when bioplastic can be emulsified into an agar matrix and bioplastic-degrading microorganisms excrete extracellular enzymes which diffuse though the agar and degrade bioplastic to water soluble materials [24]. Clear zone technique is a powerful method for ecological investigation of bioplastics degradation [19]. Sequence analysis of 16S rDNA gene of strain TF1 (1,358 bases; accession no. KC529344) showed 99% identity with *Actinomadura miaoliensis.* The various thermophilic actinomycetes are able to degrade polyesters from different environments, such as *Amycolatopsis* sp. strain K104-1[3], *Actinomadura miaoliensis* strain BC44T-5^T [4], *Actinomadura keratinilytica* strain T16-1 [5], *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2 [6], *Thermomonospora fusca* [7] and *Laceyella sacchari* LP175 [8]. Tseng *et al* [4] reported that *Actinomadura miaoliensis* strain BC44T-5^T was isolated from Miaoli country, Taiwan and had ability to degrade only PHB.

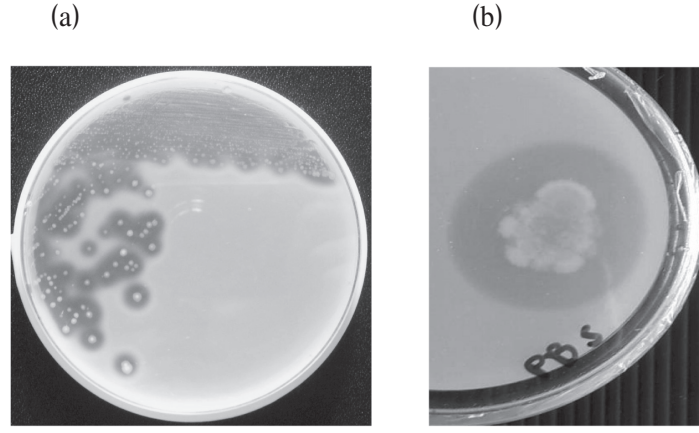

Figure 1 Colonies and clear zones formation of strain TF1 on PBS-agar plate culture at 45°C for 4 days. (a) Cross streak and (b) isolate TF1 on PBS agar plate.

Morphological and physiological characteristics of strain TF1

The morphological and physiological characteristics of strain TF1 were partially similar to *Actinomadura miaoliensis* strain BC44T-5^{T}[4] as showed in Table 1. Strain TF1 formed cream substrate mycelium on ISP2 and gray-white on ISP3 and ISP4. The color of aerial mycelium is blue-gray on ISP3 and ISP4. The temperature range for growth was 30-55°C. Strain TF1 could grow on glucose, xylose, fructose, inositol, arabinose, mannitol, raffinose and rhamnose. The strain could degrade soluble starch, caboxymethyl cellulose (CMC), avicel, xylan, skim milk and tributyrin at 45°C and produced the thermostable enzymes. Then, this strain is useful in biotechnological applications. Strain BC44T-5^{T} displayed substrate mycelia and short spore chains were borne on aerial mycelia and the spores were non-motile, round, 1 μm in diameter and spiny and growth occurred between 25-55°C. Glucose, xylose, fructose, sorbitol, rhamnose, lactose and trehalose could be utilized, but inositol, arabinose, galactose and inulin were not utilized [4].

Table 1 Comparative physiological characteristics between *Actinomadura* sp. strain TF1 and *Actinomadura miaoliensis* strain BC44T-5^T

Characteristic	***Actinomadura* sp. strain TF1**	***A. miaoliensis* strain BC44T-5^T**
Aerial hyphae colour	blue-gray	blue
Growth temperature (°C)	30-55	22-55
Growth in NaCl (%)	2-6	ND
Degradation of	CMC, avicel, xylan, soluble starch, skim milk and tributyrin, PHB, PLA, PBSA, PLLA, PCL, PBS	aesculin, casein, adenine, hypoxanthine xanthine L-tyrosine and PHB
Growth on carbon source:		
Glucose	+	+
Xylose	+	+
Fructose	+	+
Galactose	ND	-
Inositol	+	-
Inulin	ND	-
Sorbitol	+	+
Sucrose	+	+
Trehalose	ND	+
Arabinose	+	-
Mannitol	+	ND
Raffinose	+	ND
Rhamnose	+	+

+= positive, -= negative, ND = not determined

Biodegradation studies

The degradation of PBS films by strain TF1 was carried out for 90 days. After 30 days of cultivation, the PBS films started to disintegrate and at 60 days of cultivation, the fragments of the film were dispersed in the culture broth. Figure 2 showed the change in shape, strength and weight loss after incubation at 45°C in basal medium. The weight loss (%) of PBS films increased 16%, 23% and 60% of PBS film in control after incubation in 30, 60 and 90 days, respectively. In comparison with the degradation of PBS emulsion and degradation of PBS film occurred very slowly. PBS emulsion was degraded rapidly and almost completely because of its very small particle sizes and large surface area. On the other hand, the degradation of PBS film was comparatively low because of its larger particle sizes. The surface area of the polymer is an important factor in determining the rate of microbial degradation. It has been reported that crystal structure and molecular weight influence the microbial degradability of polymers [25, 26]. These observations suggested that strain TF1 could grow in PBS films in basal medium to use PBS films as carbon source and PBS depolymerase was secreted in basal medium for biodegradation process. Pranamuda *et al.* [27] studied the degradability of PCL and PBS by culture broth supernatant of an actinomycete, strain HT-6 and reported that biodegradation of these plastics occurred by hydrolysis of ester bonds. Xu and Guo [28] reported that degradation of PBS can be divided into hydrolytic degradable, enzymatic degradation and biodegradation in environmental conditions such as burial and compost. Biodegradation of PBS film is sensitive to the biodegradation conditions such as microorganisms, temperature, pH and aerobic. At pH 7.2, the weight loss of PBS is less than 10% after 9 weeks and 75% after 15 weeks [29]. The molecular weight of PBS decreases which indicates hydrolytic degradation proceeds via random chain scission [29]. Scanning electron microscopy of PBS film in basal medium for 30 days of incubation showed many changes in surface morphology such as erosion and extensive roughening of the surface with pit formation as compared to the untreated strain TF1 PBS pieces (Figure 3). Enzymatic degradation proceeds only on the surface of the PBS film occurred by both the surface erosion and weight loss because enzyme can not penetrate the polymer system of PBS film [28].

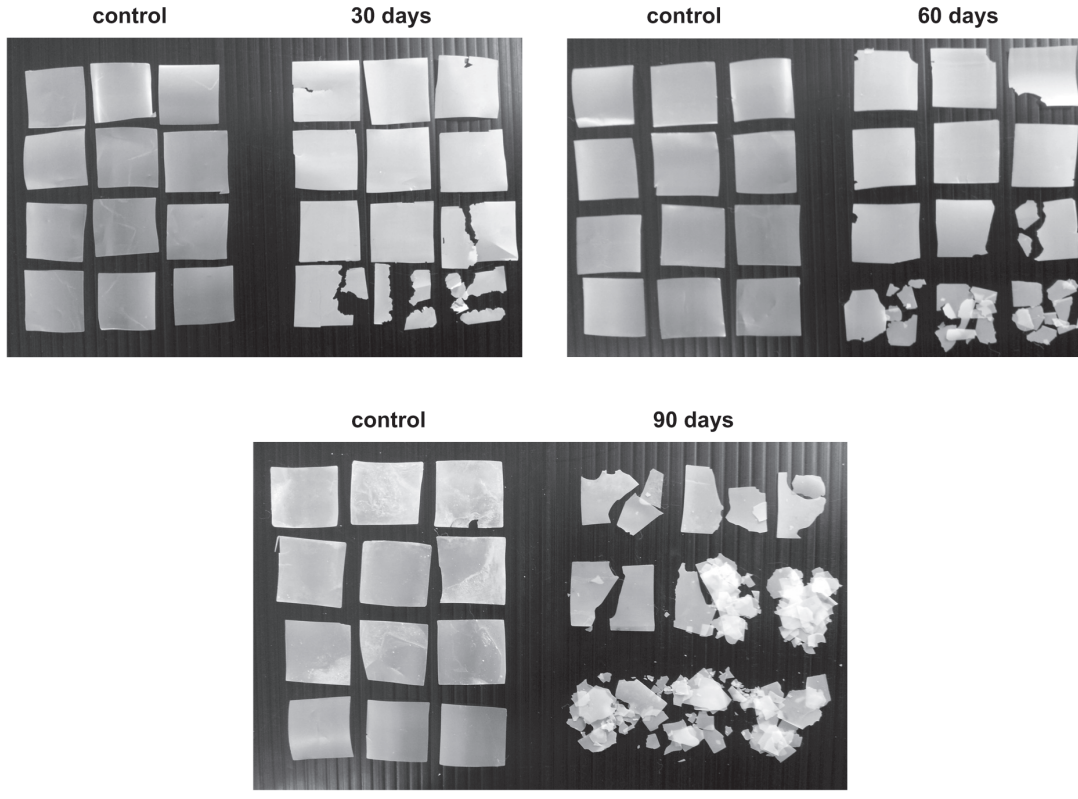

Figure 2 Photographs of PBS films degradation from strain TF1 at 30, 60 and 90 days

(a) (b)

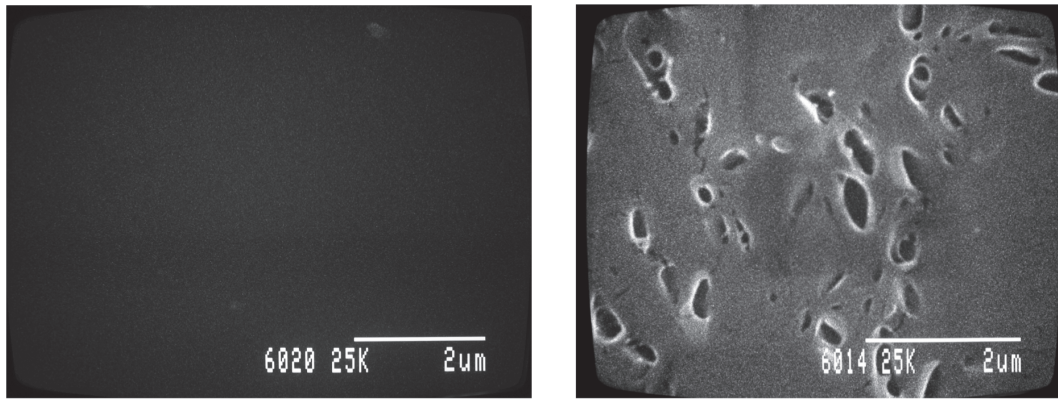

Figure 3 Scanning electron microscopy of PBS film pieces (a) control untreated strain TF1 and (b) after incubation for 30 days

Effects of pH and temperature of PBS depolymerase activity

The fourth day of crude enzyme from strain TF1 showed optimum pH 7.0 and PBS depolymerase activity was 18.2 ± 2.61 U/ml at 45°C as showed in Figure 4a. The optimum temperature was 45°C (Figure 4b). Polyester degradation is generally influenced by many factors such as polymer substance, the kind of organism involved in biodegradation and environmental conditions (e.g. temperature, pH, etc.) [30]. The optimum temperature of PBS depolymerase from *Acidovorax delafieldii* strain BS-3 was 30°C [10].

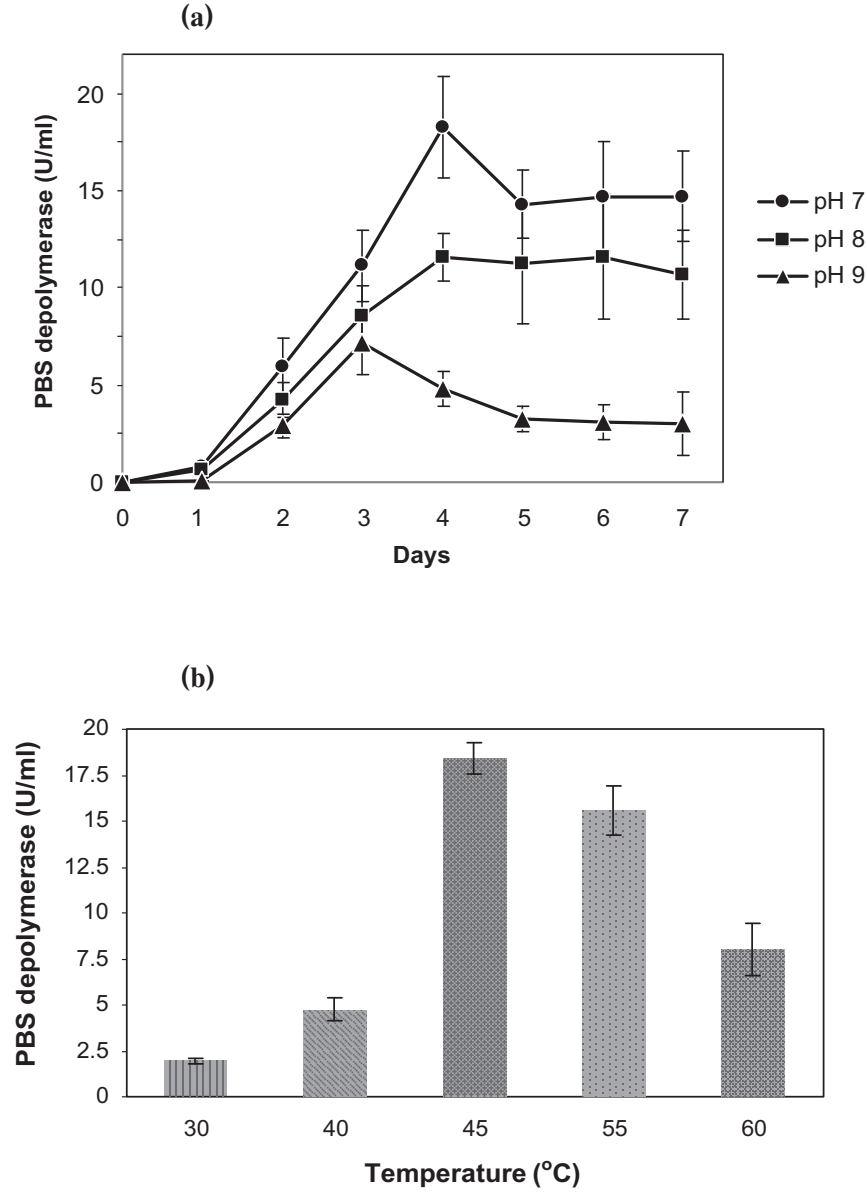

Figure 4 Effects of pH and temperature on PBS emulsion in basal medium. (a) optimal pH and (b) optimal temperature on the activity of strain TF1 PBS depolymerase

Effects of various carbon sources and nitrogen sources on cell growth

Polyesters (PLA, PBS and PBSA) were useful carbon sources for PBS degradation (Figure 5a). These results strongly suggest that PBS depolymerase from strain TF1 was induced by PBS, PLA and PBSA and was an inducible enzyme. Yeast extract with ammonium sulphate was the best nitrogen source and had the highest PBS depolymerase activity about 21.8 ± 1.96 U/ml (Figure 5b). The growth of polyester degrading microorganisms must be optimized by controlling of temperature, pH, humidity, incubation time and substrate to help in the production of large amount of enzyme [31]. In addition, the degradation potential of the different isolated organisms was greatly influenced through variation/optimization of the growth medium, since the different organisms exhibit different nutritional requirements [30].

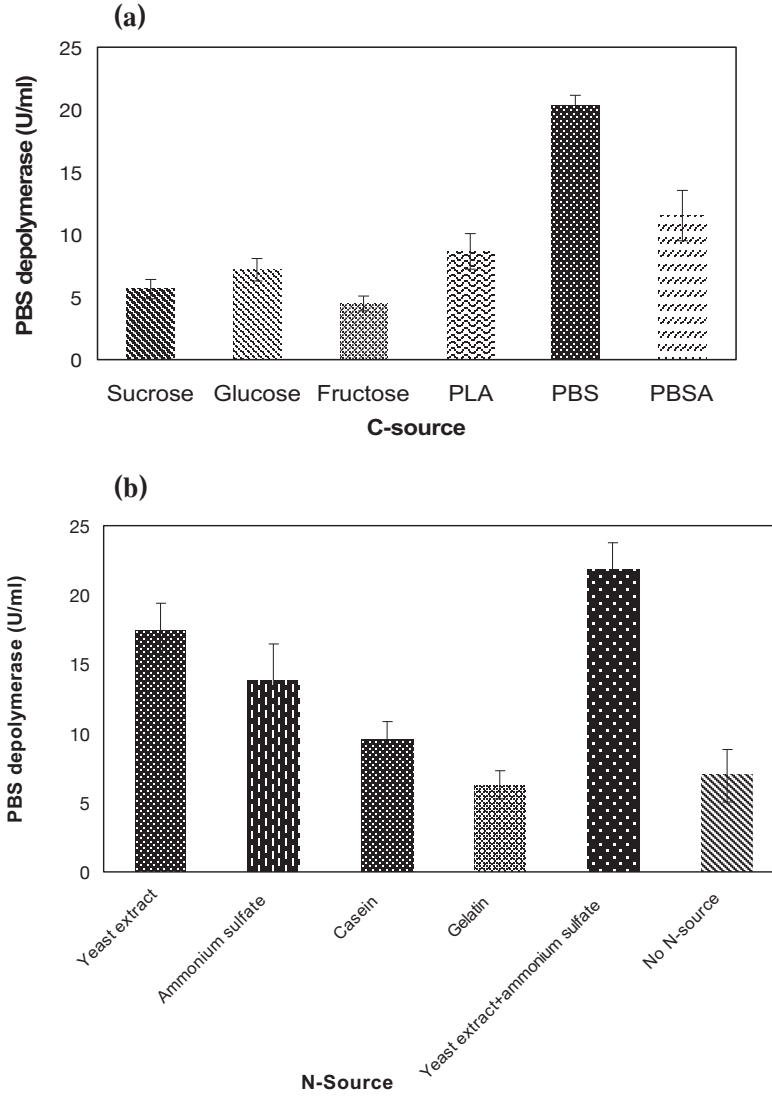

Figure 5 Effects of carbon sources and nitrogen sources on the production of PBS depolymerase from strain TF1 at 45°C for 4 days. (a) carbon sources and (b) nitrogen sources

Conclusions

PBS has found applications in agriculture, fishery, forestry and civil engineering and used as packaging materials, vegetation nets, mulching film and compost bags [28]. In this work, we reported the isolation of a thermophilic actinomycete, *Actinomadura* sp. strain TF1 capable of degrading various aliphatic polyesters. Strain TF1 could produce PBS depolymerase enzyme in PBS film and emulsified PBS in basal medium. Production of the highest PBS depolymerase activity was optimized at 45°C pH 7.0 and used PBS and yeast extract plus ammonium sulphate as carbon and nitrogen source, respectively. Thus, this strain has potential for application in biodegradable plastic composting technology. This study is the first report for the optimization of PBS depolymerase production by *Actinomadura* sp. strain TF1. However, further studies were in progress in the purification of PBS depolymerase and determined depolymerase gene in strain TF1.

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