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Production of poly-3-hydroxybutyrate from mixed culture

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Abstract: Poly-3-hydroxybutyrate (PHB) is a biodegradable polymer produced by many bacteria. Some of the properties are similar to thermoplastics like polypropylene, hence finding application PHB can directly replace non-biodegradable polymers. But the main barrier has been the cost difference. The utilization of mixed microbial cultures facilitates the use of complex substrates and thus can reduce the cost of PHB production. In the present study, mixed culture systems, where metabolite produced by one organism may be assimilated by the other organism, were employed. *Bacillus firmus* NII 0830, the first organism, was used for the production of PHB since it accumulates a large amount of PHB, while the second organism, *Lactobacillus delbrueckii* NII 0925, was used to provide acetic acid. Enzyme kinetic studies were performed on the PHB biosynthetic enzymes, such as β-ketothiolase, acetoacetyl CoA reductase and PHB synthase. PHB production by mixed culture was higher when compared to pure cultures. The mathematical model was then fitted to the experimental data, which can describe the dynamics of a mixed culture. The β-ketothiolase and acetoacetyl CoA reductase showed a V_{max} value of 0.0093 μM/min and 0.0253 μM/min, respectively. The K_m values were 140.8 μM and 183.5 μM, respectively. The enzyme kinetic studies gave an idea about the action of the enzymes.

Key words: poly-3-hydroxybutyrate; fermentation; mixed culture; biopolymer; production.

Abbreviations: DTNB, dithionitrobenzoic acid; MCB, mixed culture biotechnology; PHB, poly-3-hydroxybutyrate.

Introduction

During the past few decades, the use of plastics in packaging and other products has increased. Plastics being xenobiotic are recalcitrant to microbial degradation (Fletcher 1993). The non-degradable petrochemical plastics accumulate in the environment and high-molecular weight seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time. The environmental impact of persistent plastic wastes is evoking more global concern as alternative disposal methods are limited and this leads to considerable interest in the development of biodegradable plastics. Selection of a suitable carbon source is an important factor for reducing the production costs. Currently, the poly-3-hydroxybutyrate (PHB) is commercially produced from two genetically modified strains of *Escherichia coli* and *Alcaligenes* species. Disadvantages of PHB production from pure cultures include high costs of pure substrates utilized, costs for sterile pre-cultivation of bacteria and sterile operation for the final process (Kleerebezem & Loosdrecht 2007).

One of the main limitations for the commercial production of PHB is the production cost when compared to synthetic plastics. Several research and developmental activities have been devoted for developing better bacterial strains and more efficient fermentation and recovery processes. The use of mixed cultures and cheap substrates can reduce the cost of PHB. Accumulation of the polymer by mixed cultures occurs under transient conditions by intermittent feeding and variation in the presence of electron donor/acceptor. The maximum capacities for the biopolymer storage and production rates are dependent on the substrate and operations conditions used (Salehizadeh & Loosdrecht 2004). Using mixed culture biotechnology (MCB) the required metabolic capacities and the microbial population can be effectively enriched from a natural environment. The advantages of MCB over pure culture include no sterilization requirements, addaptive capacity owing to microbial diversity, the capacity to use mixed substrates and the possibility of a continuous process.

MCB exploits the use of mixed cultures and ecological selection principles to produce products like biopolymers from waste stream. It combines the

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methodology of environmental biotechnology with the goals of industrial biotechnology. Earlier studies revealed that MCB led to higher production of biopolymer, which is comparable or superior to pure cultures including genetically modified organisms (Johnson et al. 2009).

The objectives of the present study were to develop a strategy for utilization of mixed culture for PHB production as well as to evaluate growth kinetics, enzyme kinetics as well as additional nutrient factors affecting PHB production by mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925. Kinetic studies were carried out to find the PHB production rate, production yield and PHB content.

Material and methods

Chemicals and reagents

Crotonic acid was procured from Sigma-Aldrich, India. All other chemicals were procured from local vendors.

Microorganisms

Bacillus firmus NII 0830 and *Lactobacillus delbrueckii* NII 0925 were obtained from the NII culture collection at NIIST, Trivandrum. The stock cultures were maintained on nutrient ager slants (Shalin et al. 2014).

Culture media and inoculum preparation

Seed cultures were prepared in Luria Bertani media for *B. firmus* NII 0830 and *Bacillus sphaericus* NII 0838 so as to make an 18 h old inoculum, while for *L. delbrueckii* NII 0925 the seed culture was prepared in de Man Rogosa and Sharp medium so as to make an 18 h old inoculum (Shalin et al. 2014).

PHB assay

PHB assay was carried out by the method of Law & Slepeckey (1961).

Comparison of PHB production by mixed culture of B. firmus with B. sphaericus and L. delbrueckii

B. firmus NII 0830 inoculum (1% v/v) was added into 100 mL of minimal salt medium with glucose as sole carbon source and incubated at 200 rpm, 30 ℃ for 18 h. *B. sphaericus* NII 0838 and *L. delbrueckii* NII 0925 inocula (1% v/v) were added to separate *B. firmus* NII 0830 cultures after 18 h and incubated. Samples were taken and analysed.

Studies on the effect of supplementation of additional nutrients on growth and PHB accumulation

PHB production at different time points by mixed culture of *B. firmus* NII 0830 and *B. sphaericus* NII 0838 as well as *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 using glucose as sole carbon source followed by the supplementation of glutamate was studied. *B. firmus* NII 0830 inoculum (1% v/v) was added into 100 mL of minimal salt medium and incubated at 200 rpm, 30 ℃ for 18 h. *B. sphaericus* NII 0838 and *L. delbrueckii* NII 0925 inocula (1% v/v) were added to separate *B. firmus* NII 0830 cultures after 18 h and incubated. The media was supplemented with 2 g/L of glutamate after the 18 h of incubation. Samples were collected at 6 h intervals and analyzed.

Kinetics of growth and PHB production by B. firmus, L. delbrueckii and their mixed culture

PHB production and growth of *B. firmus* NII 0830, *L. delbrueckii* NII 0925 and their mixed culture were studied by using glucose as the sole carbon source. *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 inocula (1% v/v) were added into 100 mL of minimal salt medium with glucose as the sole carbon source and incubated at 200 rpm, 30 ℃ for 72 h. For the mixed culture, *B. firmus* NII 0830 (1% v/v) inoculum was added and *L. delbrueckii* NII 0925 inoculum (1% v/v) was added after 30 h of incubation and monitored every 12 h for a period of 72 h.

Estimation of residual glucose and acetic acid concentration

Residual glucose concentration and acetic acid concentration in the cell supernatant were estimated by HPLC (Shimadzu). As a mobile phase, the 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min was used. Aminex HPX-87H organic acid column from Bio-Rad and RI detector were used (Sluiter et al. 2008).

Growth kinetics of pure and mixed culture of B. firmus and L. delbrueckii

The growth pattern of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 and their mixed culture were studied for 72 h. Minimal salt media (100 mL) were inoculated with 18 h old culture, 1% v/v for pure cultures of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925, and 1% v/v of *B. firmus* NII 0830 and 1% v/v of *L. delbrueckii* NII 0925 at 30 h was incubated at 200 rpm at 30 ℃. The absorbances of the samples were read at 600 nm.

Enzyme kinetics

Approximately 0.2–1.0 g (wet weight) of cells was suspended in 5 mL of 100 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication for 2 min by using ultrasonic disintegrator with a probe 9.5 mm in diameter. To obtain the crude cellular extract, the unbroken cells were removed by centrifugation for 15 min at 12,000× *g* at 4 ℃. The samples were stored at –80 ℃. The protein contents in crude extracts were determined by using nano-drop spectrophotometer at 280 nm (Desjardins et al. 2009).

Enzyme assays

Acetoacetyl-CoA reductase assay. The assay was conducted according to Lynen & Wieland (1955). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 250 μM NAD(P)H, 50 μM acetoacetyl-CoA, 25% (w/v) NaCl, 0.2% (w/v) KCl, 0.5% (w/v) $MgSO_4.7H_2O$ and crude extract. The assay was started by the addition of acetoacetyl-CoA, and enzyme activity was monitored by measuring spectrophotometrically the change of absorption at 365 nm due to the oxidation of NAD(P)H to $NAD(P)^+$. The oxidation of 1 μmol NAD(P)H per min corresponded to 1 unit of the enzyme activity.

β-Ketothiolase assay. Crude extracts were subjected to the β-ketothiolase assay. The assay was conducted according to Nishimura et al. (1978). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 50 μM $MgCl_2$, 50 μM acetoacetyl-CoA, 100 μM CoA, 0.2% (w/v) KCl, 25% (w/v) NaCl and crude extract. The assay was started by the addition of CoA, and enzyme activity was monitored by measuring spectrophotometrically at 303 nm the decrease of the acetoacetyl-CoA concentration. The conversion of 1 μmol acetoacetyl-CoA to 2 acetyl CoA per min corresponded to 1 unit of the enzyme activity.

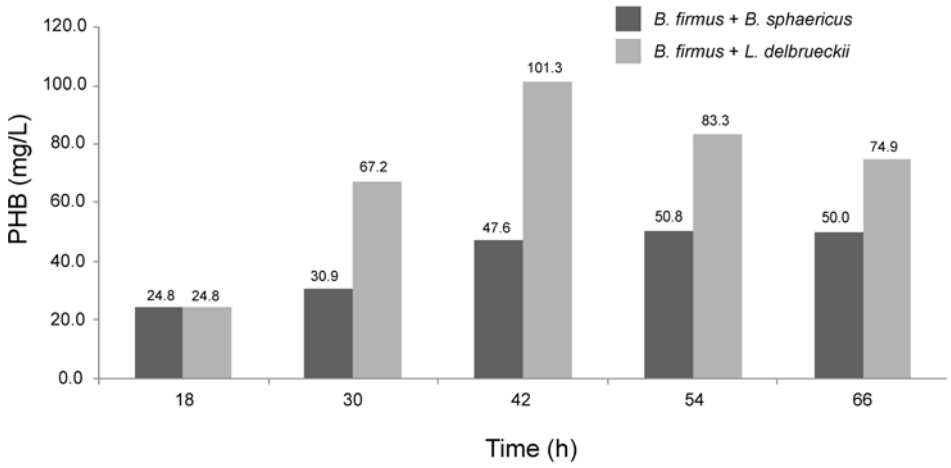

Fig. 1. Comparison of PHB production by mixed cultures.

PHB synthase assay. PHA synthase activity was monitored with a continuous assay, in which free CoA reacts with dithionitrobenzoic acid (DTNB). A 50 mM stock solution of DTNB in 0.1 M $KHCO_3$ was prepared fresh before each use. The assay mixture contained the following in a final volume of 1 mL: 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTNB and 60 mM HB-CoA. Reactions were performed at 30 °C and started by addition of 50 µL enzyme. The ΔA at 412 nm was monitored using ϵ_{412} = 13,600 $cm^{-1}M^{-1}$ for the released thiolate (Ellmann 1959). The assays were done for samples at 12 h interval up to 72 h. Both K_m and V_{max} were determined for acetoacetyl CoA reductase and β-ketothiolase from slopes of various concentrations of substrate (10-200 µM) by applying a nonlinear curve fit. Assay was continued for 60 min and readings were taken at 5 min interval for the samples at 36th h. Kinetics analysis was performed using Graph Pad Prism version 5.0 software.

Results and discussion

Comparison of PHB production by mixed culture of B. firmus with B. sphaericus and L. delbrueckii

The results presented in Figure 1 clearly indicates that, compared to *B. firmus* NII 0830 and *B. sphaericus* NII 0838 combination, *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 gave more PHB production. *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 gave 101.3 mg/L PHB after 48 h of incubation and after the addition of the second culture, whereas *B. firmus* NII 0830 and *B. sphaericus* NII 0838 gave only 47.6 mg/L. At the end of 66 h after the addition of the second culture, *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 gave 74.9 mg/L. The PHB production by *B. firmus* NII 0830 and *B. sphaericus* NII 0838 at that time was only 50 mg/L. In this mixed culture system the glucose is converted to lactate by *L. delbrueckii* NII 0925 and the lactate was converted to PHB by *B. firmus* NII 0830. An identical observation was earlier reported by Tohyama & Shimizu (1999) for mixed culture of *Ralstonia eutropha* and *Lactobacillus delbrueckii* for the production of PHB from glucose via lactate.

Studies on the effect of supplementation of additional nutrients on growth and PHB accumulation

The results presented on Figure 2 clearly indicates that there is a negative impact on the effect of supplementation of additional nutrients on growth and PHB accumulation. An identical observation was earlier reported by Meenakshisundaram et al. (1997) that glutamate can serve as a carbon source as well as a growth factor in the presence of acetate, and hence is a partially substitutable carbon source. Glutamate was supplemented additionally to each of these combinations to see if it has any effect in growth and PHB production. In the case of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925, the PHB production decreased from 24.77 mg/L to 14.63 mg/L, as well as the PHB production decreased from 24.77 mg/L to 2.7 mg/L for *B. firmus* NII 0830 and *B. sphaericus* NII 0838 combination. Deepthi et al. (2011) carried out media engineering for improved PHB production from *B. firmus* NII 0830 using biodiesel industry generated waste glycerol as sole carbon source. The study revealed that maximum PHB production, 53% of cell dry weight, was observed when crude glycerol medium was supplemented with $(NH_4)_2SO_4$ and KH_2PO_4. Jincy et al. (2013) reported an improved production of PHB from *B. firmus* NII 0830 by using biodiesel industry generated crude glycerol by adopting a response surface strategy. Under optimized conditions 1.6 g/L of PHB was observed at inoculum size of 3% v/v, incubation temperature 30 °C, crude glycerol concentration 5% v/v, 250 rpm, incubation time 60 h and media pH 6.0.

Growth kinetics of pure and mixed culture of B. firmus and L. delbrueckii

Figure 3 depicts the growth pattern of *B. firmus* NII 0830, *L. delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 that was studied when glucose was given as the carbon source. Mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 showed good growth reaching up to 2.9 mg/mL, while *L. delbrueckii* NII 0925 exhibited very less growth with only 1.4 mg/mL after 30 h of growth

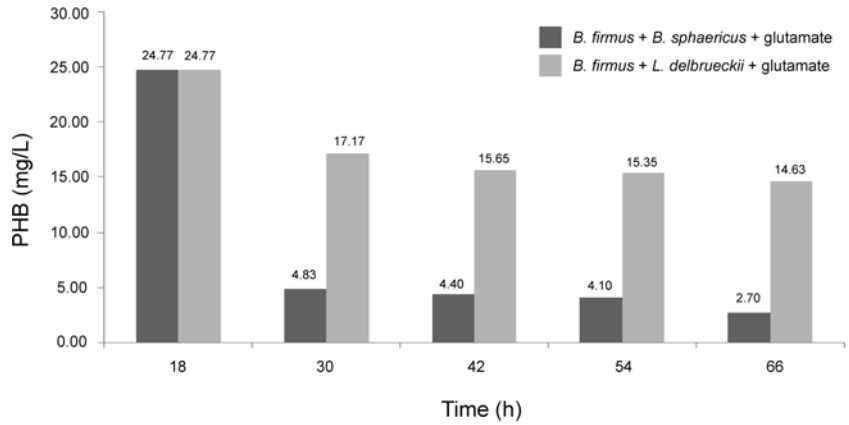

Fig. 2. PHB production and growth profile by mixed culture when glutamate was supplied as an additional nutrient.

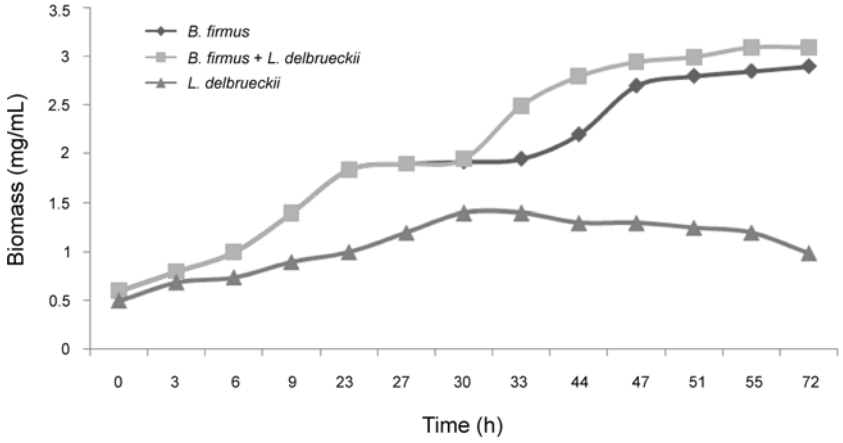

Fig. 3. Growth profile of *Bacillus firmus* NII 0830, *Lactobacillus delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925.

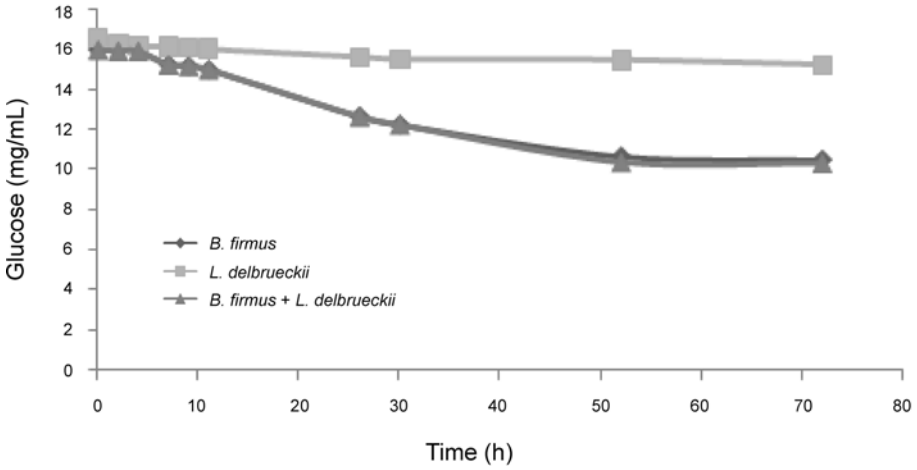

Fig. 4. Glucose consumption by *Bacillus firmus* NII 0830, *Lactobacillus delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925.

and then decreased and reached 0.99 mg/mL at the end of 72 h. While *B. firmus* NII 0830 showed good growth reaching up to 2.7 mg/mL after 72 h of incubation.

The substrate consumption, PHB production and acetate formation by *B. firmus* NII 0830, *L. delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 were also monitored. Figure 4 depicts the glucose consumption profile of *B. firmus* NII 0830, *L. delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925. The results indicate that for *B. firmus* NII 0830 the substrate consumption was higher, i.e. it decreased to a level of 10.46 mg/mL, whereas in the case of *L. delbrueckii* NII 0925 the substrate consumption was only

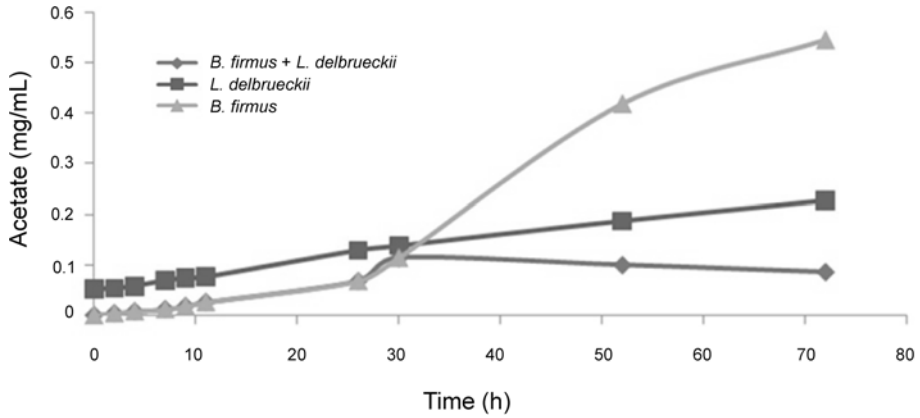

Fig. 5. Acetate production profile by *Bacillus firmus* NII 0830, *Lactobacillus delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925.

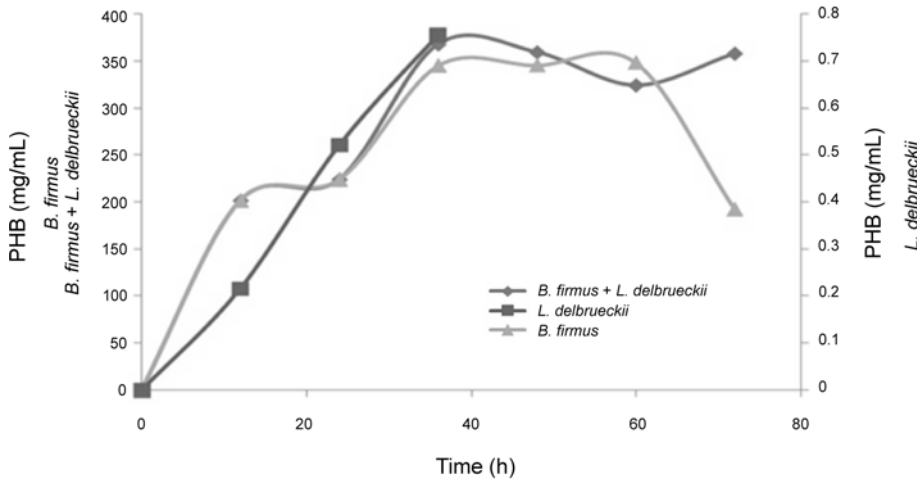

Fig. 6. PHB production profile by *Bacillus firmus* NII 0830, *Lactobacillus delbrueckii* NII 0925 and the combination of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925.

up to 15.22 mg/mL. While the mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 showed substrate consumption up to 10.33 mg/mL.

Comparison of acetate production by pure culture of B. firmus, L. delbruekii and their mixed culture

Figure 5 illustrates the acetate production profile of pure culture of *B. firmus* NII 0830 and *L. delbruekii* NII 0925 as well as their mixed culture. More acetate formation was observed in the case of *L. delbrueckii* NII 0925 than for *B. firmus* NII 0830 with a maximum concentration of 0.546 mg/mL and 0.227 mg/mL, respectively. In the case of mixed culture, after the addition of the second culture the acetate concentration was decreased to 0.114 mg/mL after 72 h of incubation. Acetate was quickly taken up and stored as PHB. Acetate consumption and PHB production was linear during the feast period. An identical observation was earlier reported for PHB production by mixed culture by Serafim et al. (2004).

Comparison of PHB production by pure culture of B. firmus, L. delbruekii and their mixed culture

Figure 6 shows the PHB production profile of pure culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 as well as their mixed culture. *B. firmus* NII 0830 produced 348.07 mg/mL PHB and the PHB production by *L. delbrueckii* NII 0925 was negligible amounting to only 0.75 mg/mL. The mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 gave a maximum of 367.4 mg/mL after 60 h of incubation.

Enzyme kinetics

Figure 7 depicts the changes of activities of the three PHB biosynthetic enzymes: β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase during cultivation. The activities of the three PHB biosynthetic enzymes increased gradually with increase of incubation time. The maximum activities of β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase were 2.5 U, 1.25 U and 1.25a U, respectively. The maximum activity of β-ketothiolase was observed at 40 h, while for acetoacetyl-CoA reductase and PHB synthase they were observed around 24 h.

During cultivation period where the PHB accumulation ceased, all the enzyme activities related to PHB biosynthesis started to decrease. On the other hand, activity of β-ketothiolase remained at a comparatively

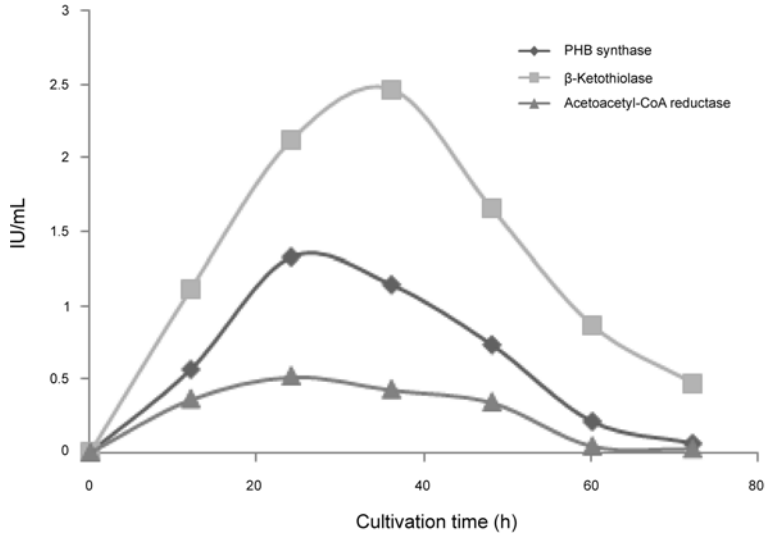

Fig. 7. Kinetics of PHB biosynthetic enzymes.

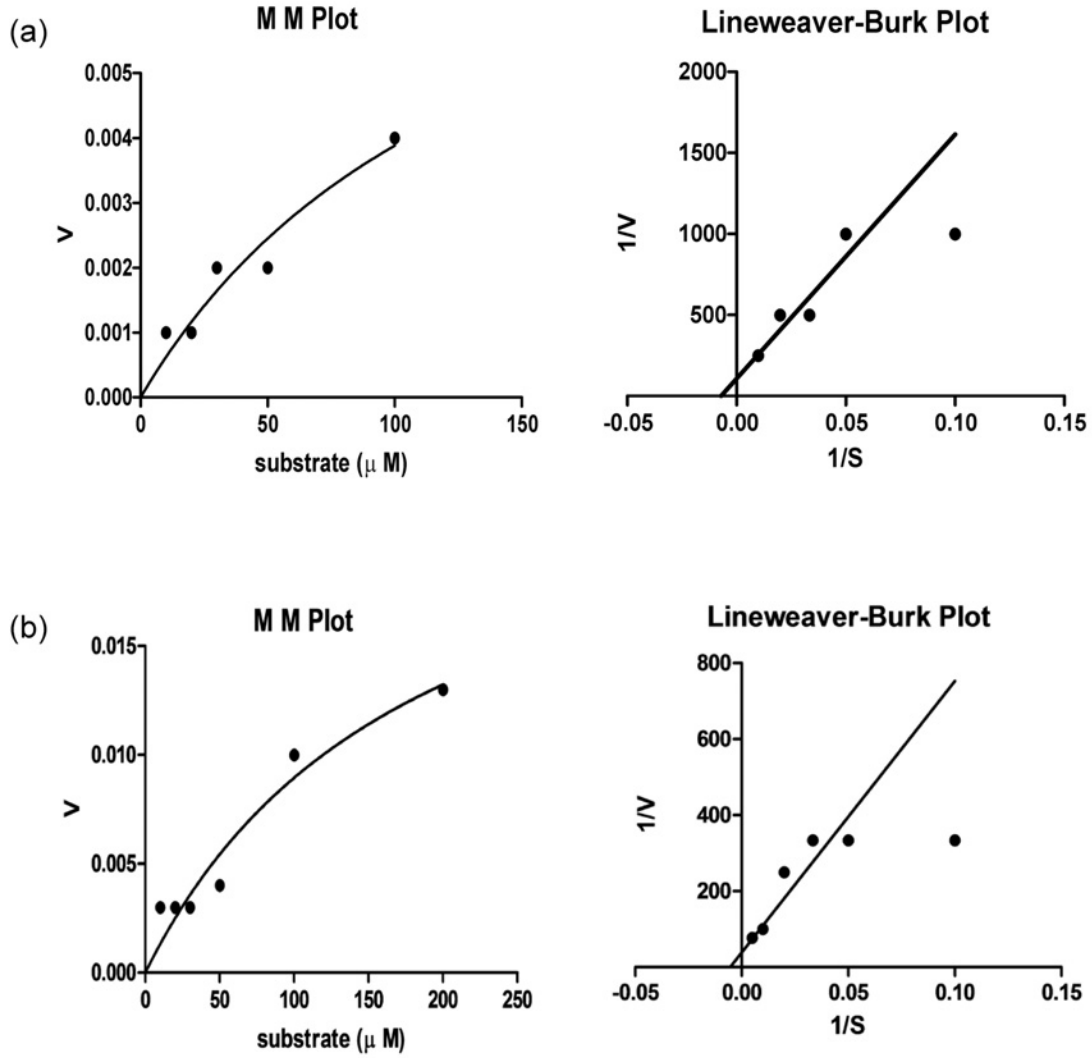

Fig. 8. Michaelis-Menten (MM) and Lineweaver-Burk plots for β-ketothiolase (a) and acetoacetyl CoA reductase (b).

higher value. The higher level of residual activity of β-ketothiolase can be explained by the special dual functions of β-ketothiolase in that it participates not only in PHB biosynthesis, but also in the PHB degradation reaction that converses acetoacetyl-CoA to acetyl-CoA for starting material for the tricarboxylic acid cycle.

Kinetic studies were performed for the PHB biosynthetic enzymes β-ketothiolase and acetoacetyl-CoA reductase. The protein content of the sample was 22 mg/mL. K_m and V_{max} were determined for acetoacetyl CoA reductase and β-ketothiolase from slopes of various concentrations of substrate (10-200 μM) by applying a nonlinear curve fit. β-Ketothiolase gave K_m and V_{max} values of 140.8 μM and 0.0093 μM/min, respectively (Fig. 8a), whereas acetoacetyl CoA reductase gave K_m and V_{max} values of 183.5 μM and 0.0253 μM/min, respectively (Fig. 8b).

Conclusions

One of the major limitations for the commercial production of PHB is the lack of a cost effective production technology. This can be overcome to a certain extent by using cheap carbon source as well as by using mixed culture. Several research and developmental activities are going on in this direction. The present study revealed that more PHB was produced by a mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 than by their. Enzyme kinetics studies of β-ketothiolase and acetoacetyl CoA reductase documented that the major PHB biosynthetic enzymes gave a better understanding of the action of enzymes and their role in PHB production. All the three enzymes, i.e. β-ketothiolase, acetoacetyl CoA reductaseand PHB synthase showed the highest activity after 36 h of incubation time, where active PHB synthesis takes place. Mixed culture system in the present study will be promising as far as the culture conditions are properly controlled. Fine tuning of the process would make it economically viable.

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