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ORIGINAL PAPER

Starch based polyhydroxybutyrate production in engineered *Escherichia coli*

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Abstract Every year, the amount of chemosynthetic plastic accumulating in the environment is increasing, and significant time is required for decomposition. Bio-based, biodegradable plastic is a promising alternative, but its production is not yet a cost effective process. Decreasing the production cost of polyhydroxyalkanoate by utilizing renewable carbon sources for biosynthesis is an important aspect of commercializing this biodegradable polymer. An *Escherichia coli* strain that expresses a functional amylase and accumulate polyhydroxybutyrate (PHB), was constructed using different plasmids containing the amylase gene of *Panibacillus* sp. and PHB synthesis genes from *Ralstonia eutropha*. This engineered strain can utilize starch as the sole carbon source. The maximum PHB production (1.24 g/L) was obtained with 2 % (w/v) starch in M9 media containing 0.15 % (w/v) yeast extract and 10 mM glycine betaine. The engineered *E. coli* SKB99 strain can accumulate intracellular PHB up to 57.4 % of cell dry mass.

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Introduction

Globally, there is a keen interest in adapting bio-plastics, such as polyhydroxyalkanoates (PHAs), to replace petrochemical-based plastics, due to dwindling petroleum resources and the desire to create an economical and eco-friendly alternative to plastics [1]. Polyhydroxybutyrate (PHB) is the most common type of PHA synthesized and accumulated by microorganisms in response to conditions of physiological stress [2–4]. The production of PHB is a costly process, and that cost can be decreased by developing strains that have improved fermentation capabilities and the potential to utilize inexpensive carbon sources [5, 6]. PHB production from *Escherichia coli* has been attracting attention, although, *E. coli* does not produce PHB naturally. Recombinant DNA technology and metabolic engineering make it possible to create *E. coli* strain having the potential to accumulate PHB [7]. The biosynthesis of PHB requires three reactions mediated by these enzymes: β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHB polymerase (PhaC) [8].

In large-scale production of PHB, glucose is considered as a suitable substrate for higher PHA (%) accumulation. This process is still not economical as raw material and recovery process costs make up more than 45 % of the total production cost [1]. However, the increasing market demand suggests that glucose may no longer be economically feasible as a raw material and to reduce the costs of raw material for PHB production, low cost carbon sources were recommended. There are a number of research studies devoted to identifying a suitable substrate with low costs.

Starch is a renewable carbon source available in large quantities, and prior to fermentation, it is hydrolyzed to glucose by liquefaction and saccharification, both of which add cost to production processes [9]. Wild-type *E. coli* on its own has no potential to utilize starch as carbon source and concomitantly accumulate PHB. There is a need to further improve the potential of *E. coli* strains for utilization of other abundantly available carbon source, i.e. starch, cellulose and lignocelluloses. In this study, we have constructed a bacterial strain of *E. coli* SKB99, harboring plasmids pTAmyl and pLW487, containing genes for starch hydrolysis and PHB synthesis (Table 1). Amylase hydrolyzed the soluble starch, which is further used as a carbon source for growth and PHA produced by the engineered *E. coli* strain SKB99.

Materials and methods

Microorganism and plasmids

The *E. coli* strain and plasmids used in this study are listed in Table 1. Plasmid pTAmyl, containing α-amylase gene from *Panibacillus* sp. driven by an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter and plasmid pLW487, containing PHA synthesizing genes of *Ralstonia eutropha* also driven by an IPTG-inducible promoter, were previously constructed and used by our laboratory in prior works [3, 10]. The *E. coli* strain SKB99 was prepared by transforming these plasmids (pTAmyl and pLW487) into *E. coli* BL21(DE3). Engineered *E. coli* SKB99 and parental *E. coli* BL21(DE3) cells were grown in 10 mL of M9 media, containing 2 % (w/v) starch, 0.15 % (w/v) yeast extract, 10 μM IPTG and 10 mM glycine betaine, for 96 h at 30 °C and analyzed for biomass production and PHB accumulation. All the experiments were performed in duplicates.

Analytical method

Gas chromatography, using a slight modification of a method described previously, was used for detection and quantification of PHB [3]. Approximately 10 mg of freeze-dried cells from each experiment were weighed and placed in Teflon-stoppered glass vials. For methanolysis of PHB samples, 1 mL chloroform and 1 ml methanol/H_2SO_4 (85:15 v/v) were added to the vials and incubated at 105 °C for 2 h, cooled to room temperature and incubated on ice for 10 min. After adding 0.5 mL of ice cold water, the samples were thoroughly mixed by vortex for 1 min, and then centrifuged at 2000×*g*. The organic phase (bottom) was extracted by pipette and moved to clean borosilicate glass tubes containing Na_2SO_4. These samples were then injected into a gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a fused silica capillary column (Supelco SPB-5, 30 m × 0.32 mm, i.d. 0.25 μm film) with hydrogen as the carrier gas. A 2 μL portion of the organic phase was injected using the auto sampler. The inlet was maintained at 250 °C. The oven temperature was maintained at 80 °C for 5 min, further heated to 220 °C at 20 °C min^{-1} and then held at 220 °C for 5 min. Peak detection was performed by a flame ionization detector, which was maintained at 300 °C.

Examination of amylase activity and PHB production

The *E. coli* strain SKB99 was examined for its amylase activity (starch agar plate hydrolysis method), growth and polyhydroxybutyrate production using starch as carbon source. SKB99 and the parental strain BL21 (DE3) were streaked on LB agar plates containing 1 % starch and incubated at 30 °C overnight. Resulting colonies were flooded with 2 mL iodine solution (3 g of iodine crystals and 15 g of potassium iodide in 1 L of water) and observed for starch hydrolysis (i.e., α-amylase) activity.

Engineered *E. coli* SKB99 and parental *E. coli* were grown in M9 media as mentioned above, for 96 h at 30 °C. Samples were withdrawn at 24 h intervals and analyzed for cell dry mass (CDM) and PHB production as described previously [3].

Table 1 Strains and plasmid used in this study

Strain, plasmid	Relevant information	Source and references
BL21(DE3)	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm*	Novagen
SKB99	BL21(DE3) containing plasmid pTAmyl and pLW487	This work
pLW487	Spectinomycin-resistant pEP2-based plasmid carrying PCR products of *bktB, phaB* and *phaC* from *R. eutropha* expressed under the trc promoter	[3]
pET24ma	p15A replication origin, T7 lac promoter, C-terminal 6×His-tagged coding, kan-R	[3]
pTAmyl	pET24ma carrying PCR product of *amy1* gene from *Paenibacillus* sp.	[10]

Starch, nitrogen and IPTG concentration

Concentration of starch was optimized for biomass and PHB production by varying from 0.5 to 2.5 % (w/v). Nitrogen source concentration was also optimized by varying the yeast extract concentration from 0.05 to 0.25 % (w/v). IPTG concentration has an important role in the induction of gene expression, so its concentration was optimized from 5 to 80 μM. As *E. coli* SKB99 has slow growth and PHA accumulation on starch, we have found that expression of amylase as inclusion body may be a limiting factor which hinders the secretion of amylase. To overcome this problem different osmoprotectant, i.e. ethanol 3 %, sucrose 4 %, glycine betaine 10 mM and NaCl 4 % were added in the growth media during the culture of *E. coli* SKB99. After 96 h, cells were separated from the broth and analyzed for CDM, PHA and inclusion body. For inclusion body analysis, 1 mL of cell culture centrifuged and the cell pellet was lysed using BugBuster® Master Mix from Novagen. The lyzed cell mixture was centrifuged and the supernatant was used to study soluble amylase fraction and cell palate was further used for inclusion body analysis. To study the effect of the different osmoprotectant on protein expression, SDS-PAGE analysis (staining with coomassie brilliant blue) was performed using soluble protein fraction (supernatant) and inclusion body (cell pellet).

Results and discussion

Construction of *E. coli* strain for PHB production from starch

Starch is the second most abundant source of carbon, so worldwide interest has been stimulated to use this economical carbon source in the production of a variety of value added products, i.e. bioethanol, maltose syrup, and others [11–13]. The purpose of our research was to construct a new bacterial strain that has the capacity to hydrolyze starch and use it as the sole source of carbon for growth and PHB production. Wild-type *E. coli* strain has no starch hydrolyzing enzymes (Fig. 1), so it cannot utilize starch as carbon source and as a result, it fails to accumulate PHB. Engineered *E. coli* strain SKB99, exhibiting amylase activity (Fig. 1) was able to utilize starch as a carbon source and grew steadily on the carbon source until after 72 h, during which, it likely attained stationary phase with an overall CDM of 1.15 g/L (Fig. 2) and a total polymer accumulation of 0.4 g/L PHB. In engineered *E. coli* strain SKB99, accumulation of PHB starts with the growth of *E. coli* and remains consistent until it attain stationary phase, which represents that PHB production in

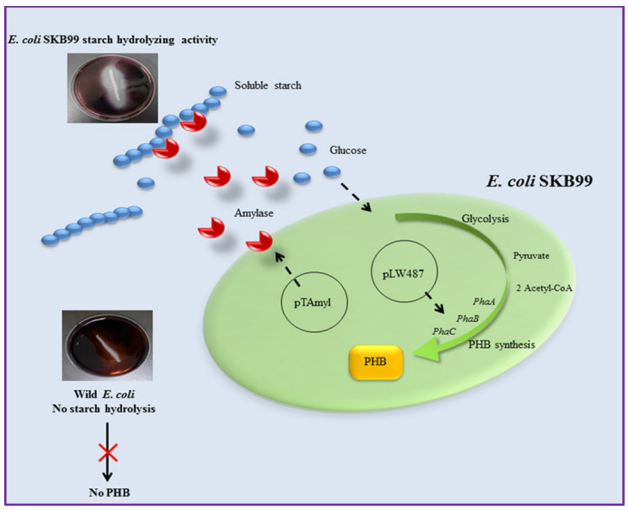

Fig. 1 Scheme for PHB production from starch. A clear zone on a LB starch agar plate is representing amylase activity in *E. coli* SKB99, but not wild-type

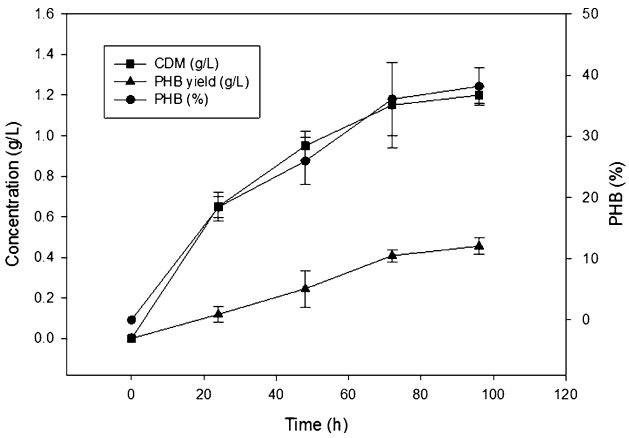

Fig. 2 Growth and PHB production profile of engineered *E. coli* SKB99 on starch as sole carbon source. Cells were grown in 50 mL of M9 media, containing 2 % (w/v) starch, 0.15 % (w/v) yeast extract and 10 μM IPTG, for 96 h at 30 °C

engineered *E.coli* is not regulated by the stress response unlike in *R. eutropha* and other microorganisms [11]. On further increase of incubation time above 72 h, there is no observed increase in residual biomass, but slight increase in accumulation of PHB was recorded, which might be due to depletion of nitrogen source required for growth and access of carbon source in the media (Fig. 2).

Effect of starch and nitrogen concentration

As starch was used as the sole carbon source, its concentration in the growth media had an effective role in biomass production and accumulation of PHB in *E. coli* SKB99. PHB concentrations increased with the increase of biomass production, and 0.55 g/L PHB was recorded after 72 h

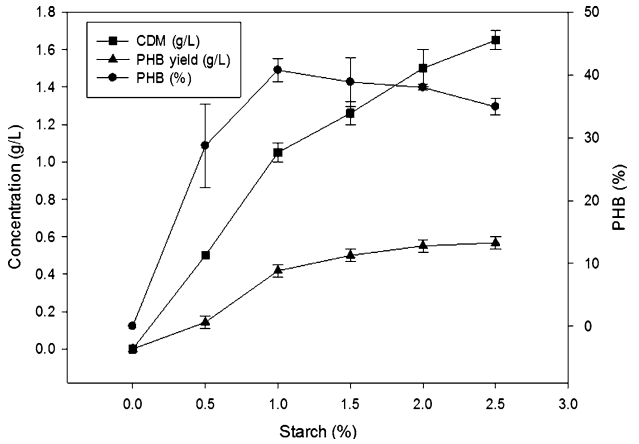

Fig. 3 Effect of starch concentration on PHB accumulation in *E. coli* SKB99. Cells were grown in 10 mL of M9 media, containing different concentrations of starch (0–3 %)

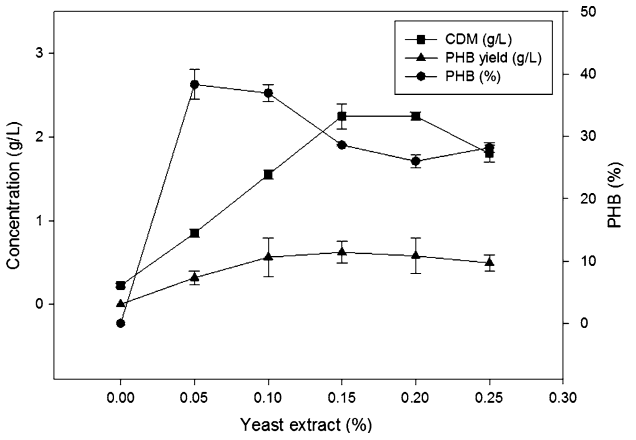

Fig. 4 Effect of yeast extract concentrations on PHB accumulation. The cells were grown in 50 mL of M9 media, containing 2 % (w/v) starch, different concentration of yeast extract(0–0.3 %) and 10 μM IPTG, for 96 h at 30 °C

using 2 % (w/v) starch concentration in the growth media (Fig. 3). Upon increase of starch concentration (1.0–2.5 %), there is an observed decrease in the intracellular accumulation of PHB (40–34 %), because the rate of increase of residual biomass production was higher in comparison to the rate of PHB production at a higher starch concentration (Fig. 3). Yeast extract is commonly reported as a nitrogen source for *E. coli* growth [3]. Optimum concentration of yeast extract was demonstrated to be 0.15 % (w/v), with 2.25 g/L of CDM and 0.62 g/L PHB accumulations recorded at this level of nitrogen (Fig. 4). In previous studies, 0.1 % yeast extract was used as a nitrogen source for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) production using a different engineered *E. coli* strain [3]. With the increase of yeast extract concentration, there was an observed increase in CDM and overall PHB concentrations, but the rate of increase in CDM is higher as compared to PHB accumulation, so an overall decrease in PHB (% of CDM) was observed with the increase of yeast extract concentration. Thus, carbon and nitrogen source have an effective role in biomass production and accumulation of PHA. Carbon and nitrogen ratio of 2:0.15 proved as effective for optimum biomass production and PHA accumulation. As mentioned previously, accumulation of PHA in *E. coli* is independent of nutrient concentration and it starts to accumulate PHA as the cell grows. Furthermore, *E. coli* is free of PHA degrading enzymes which resulted into enhanced PHA accumulation [11]. This is the first report on PHB production using starch as carbon source, although there are a number of reports on the production of PHA by recombinant *E. coli* using glucose, molasses and whey as carbon sources [14–17].

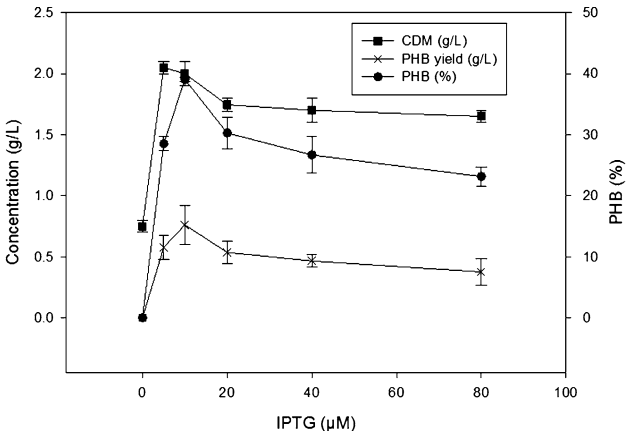

Fig. 5 Effect of inducer (IPTG) concentration on PHB accumulation in *E. coli* SKB99 grown on starch as the sole carbon source

Optimization of protein expression

Optimization of IPTG concentration is important to increase the expression of heterologous genes under control of inducible promoters in *E. coli* and to reduce the cost of the bioprocess. Optimum concentration of IPTG was recorded as 10 μM, and using this concentration, 2.0 g/L of CDM and 0.76 g/L accumulations of PHB was recorded (Fig. 5). IPTG concentration has an effective role in PHA accumulation as previously been reported for P(HB-*co*-HV) copolymer production [3]. Due to the changes in the IPTG levels, expression of proteins unneeded for growth diverts cellular resources from making necessary protein and leads to a reduction in the growth rate of an organism [18].

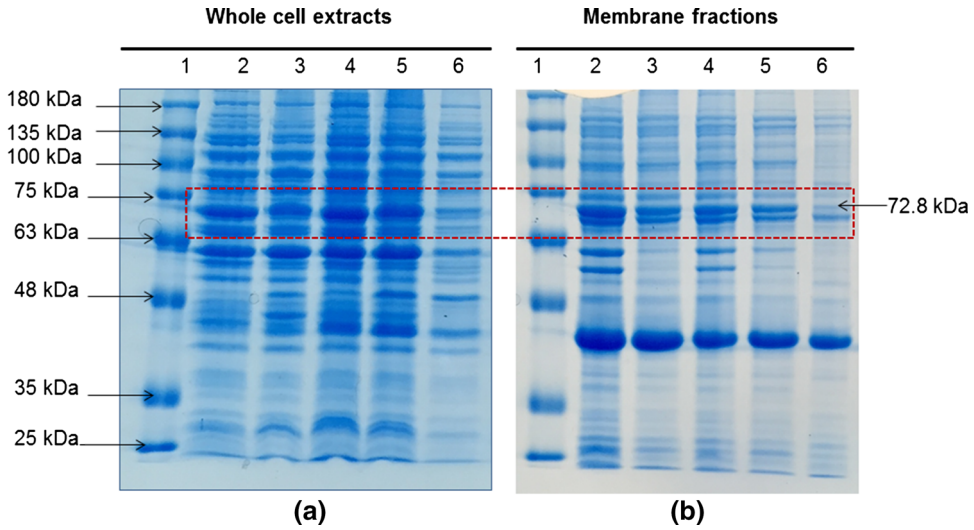

Fig. 6 SDS-PAGE showing the effect of osmoprotectant (ethanol 3 %, sucrose 4 %, glycine betaine 10 mM and NaCl 4 %) on expression of amylase (72.8 kDa). **a** Whole cell extract showed total soluble protein fraction, **b** membrane fraction representing, insoluble protein fraction of different culture obtained after growing *E. coli* SKB99 in presence of various osmoprotectant, *lane 1* marker, *lane 2* control, *lane 3* ethanol, *lane 4* sucrose, *lane 5* glycine betaine and *lane 6* NaCl

Osmoprotectants effect on PHA accumulation

Overexpression of recombinant proteins in *E.coli* mostly leads to misfold and accumulation as soluble aggregates or inclusion bodies. Different osmoprotectants were tried to increase the fraction of expressed proteins in soluble form. SDS-PAGE analysis results confirmed the change in PHA accumulation as there was an increase in the soluble amylase fraction (72.8 kDa, whole cell extract, Fig. 6a) and decrease in inclusion body of amylase (membrane fraction) was observed in the presence of osmoprotectant (Fig. 6b). An increase in the soluble amylase fraction was recorded, i.e. 18.2 and 8.1 % for glycine betaine and sucrose, respectively. Glycine betaine proved as efficient osmoprotectant and increased secreted amylase fraction (14.3 %), which resulted in a 17 % increase in PHA accumulation without effecting biomass. Sucrose, a non metabolizable sugar for *E.coli* also have a positive effect and increase 5 % PHA accumulation while ethanol and NaCl have a negative effect (Fig. 7). Osmoprotectants have very little effect on growth. Under the optimized set of condition with 10 mM glycine betaine as the osmoprotectant, PHA accumulation process was performed at 100 mL scale and 57.4 % PHA accumulation was recorded with 1.24 gL^{-1} productivity. Engineered *E. coli* strain SKB99 can accumulate up to 57.4 % of its CDM as PHB on starch, while 41.3 and 47.3 % PHB accumulation was shown in other recombinant *E. coli* strains grown on glucose and glycerol as the carbon source, respectively [19, 20]. The main advantage of this bioprocess is the utilization of starch as carbon source which is a renewable and second most abundant carbon source [11]. The effective role of the various osmoprotectant (polyol, betaine, NaCl and ethanol) for expression of recombinant protein in *E.coli* has been explored for better protein expression [21–23].

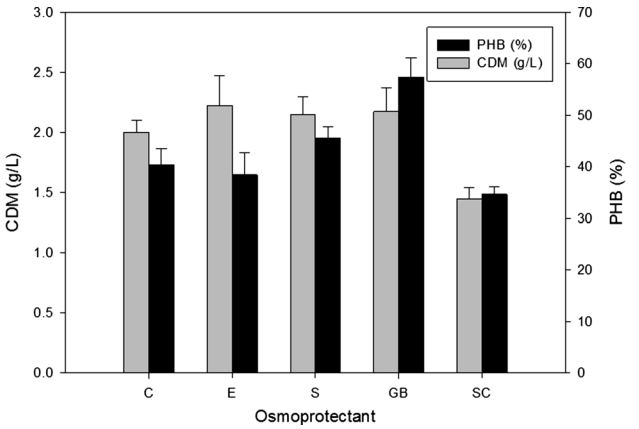

Fig. 7 Effect of different osmoprotectant on PHA accumulation and biomass production. *Different symbols* used to represent various osmoprotectants as: control (*C*), ethanol (*E*), sucrose (*S*), glycine betaine (*GB*) and sodium chloride (*SC*)

Conclusions

Production of PHA using various microbes and *E. coli* from various carbon source has been known for several years, its production using starch has not been explored yet. The results obtained in this study showed that engineered *E.coli* has the potential to utilize starch, the second most abundant carbon source, for PHA production. Thus, the result provided here presents a new approach for commercially valuable product and addition of the osmoprotectant during the culture condition enhanced soluble protein fraction which can increase the productivity.

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