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Simultaneous Production of Biopolymer and Biosurfactant by Genetically Modified *Pseudomonas Aeruginosa* UMTKB-5

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Abstract. The simultaneous production of two biotechnologically important biomaterials, poly(3-hydroxybutyrate) [P(3HB)], a well-known biopolymer and rhamnolipid (RL), a type of biosurfactant in a single culture medium have been evaluated. Both extracellular RL and the intracellular P(3HB) were produced from renewable resources by a genetically modified marine strain *Pseudomonas aeruginosa* UMTKB-5. The wild-type which was only able to produce RL was transformed by inserting plasmid pBBR-PC_{1020} harboring polyhydroxyalkanoate (PHA) synthase gene of *Cupriavidus* sp. USMAA1020, a well-known P(3HB) producer in order to produce RL as well as P(3HB). Various renewable carbon sources were used in this study such as simple sugars, oleo-chemical industry and sugar cane refinery by-products at a fix concentration of 20 g/L. The RL production by wild-type strain ranged from 54 to 272 mg/L, while the transformant was able to produce 40 to 56 mg/L. Production of P(3HB), the most common type of PHA, by the *P. aeruginosa* UMTKB-5 transformant was determined using gas chromatography and the strain was found to produce P(3HB) in the range of 9 to 24 (wt%). No P(3HB) were produced by wild-type from all the substrates. The highest P(3HB) content was detected using glycerol as carbon source in the genetically modified *P. aeruginosa* UMTKB-5. Conversely, a lower concentration of RL was produced by the transformant compared with wild-type strain. This may be due to the channeling of intermediate substrates for P(3HB) production as well as RL. This study reports the potential production of two biotechnologically important materials using bacterial fermentation in a single cultivation medium and carbon source.

Keywords: Poly (3-hydroxybutyrate), rhamnolipid, *Pseudomonas aeruginosa* UMTKB-5, glycerin pitch, glycerol, molasses, sweet water

1. Introduction

Several species from the genus Pseudomonas are known to synthesize biomaterials such as rhamnolipids (RL) as well as polyhydroxyalkanoate (PHA). The production of these biomaterials occurs in the presence of excess carbon source and limitation of nitrogen or multivalent ions [1]. *Pseudomonas aeruginosa* has been proposed by scientist as the model for rhamnolipid and medium-chain-length-PHA (MCL_{PHA}) study [2]. Other strains such as *Pseudomonas chlororaphis* and *Pseudomonas alcaligenes* have also been investigated for rhamnolipid production [3], [4]. *Pseudomonas putida* was able to produce MCL_{PHA} when saponified palm kernel oil used as substrate [5], [6].

RLs which belong to the glycolipids class are the most well documented biosurfactant. RL is composed of rhamnose sugar molecules and β-hydroxyl alkanoic acids. The extracellular rhamnolipid produced by *P. aeruginosa* in the culture medium are mainly rhamnosyl-β-hydroxydecanol-β-hydroxydecanoate (mono-RL) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-RLs) [7]. RL biosynthesis generally

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involved three major parts, which are biosynthesis of lipid moiety, biosynthesis of sugar moiety and enzymatic reactions. Biosurfactants are non-toxic, biodegradability, ecological acceptability and easily produced using renewable carbon sources [8]. Due to high potent detergent properties, biosurfactant has been applied in environment for bioremediation, dispersion of oil spill and enhance the biodegradation of hydrocarbons [9].

PHA is among the most widely investigated group of biodegradable polymer. It is produced intracellularly by various types of bacteria including *Cupriavidus* sp., *Aeromonas* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp. and recombinant *Escherichia coli* [10]. Among the different strains tested, *Cupriavidus necator* has been studied most extensively due to its ability to accumulate large amount of PHA from simple carbon sources [11]. Poly (3-hydroxybutyrate) [(P3HB)], a short-chain-length-PHA is the most common type of PHA produced by bacteria in the natural environment. PHA is synthesized by bacteria under unbalanced growth conditions such in the presence of high concentrations of carbon and limited concentration of N, P, S or some trace elements [12]. The physical properties of P (3HB) is remarkably similar to some conventional plastics [13]. This attribute has attracted much interest for P (3HB) to be widely used in packaging, medical, insecticides, herbicides, cosmetic world and disposable personal hygiene [13].

The cost of carbon source, fermentation strategy and recovery process/downstream processing contributes to the high manufacturing cost for production of PHA and RL, thus making their use unattractive [12]. In general, both PHA and RL are produced in separately, using different culture medium, different strains and fermentation conditions. This study highlights the possible production of both biomaterials using a single strain in a single culture medium under same culture conditions. Besides, this study also focuses on the use of renewable carbon sources, particularly from by-products from oleochemical industry (glycerol and glycerin pitch) and sugar cane refinery (molasses and sweet water) for the production of these biomaterials.

2. Methods

2.1. Bacterial strains, plasmids and growth condition

The bacterial strains and plasmid used in this study are listed in table 1. *Escherichia coli* was grown at 37 ℃ in Luria-Bertani medium (Himedia, India), while wild type *P. aeruginosa* UMTKB-1 was grown at 30 ℃ in nutrient rich (NR) medium consisting of the following components: per liter; 10 g peptone, 10 meat extract; 2 g yeast extract [14]. For P(3HB) and RL biosynthesis, various carbon sources, namely glucose, fructose, sucrose, glycerol, glycerin pitch, molasses and sweet water at a concentration of 20 g/L were applied.

Table 1: Bacterial strains and plasmids used in this study.

Bacterial strains and plasmid	Relevant phenotype	Source of references
Bacterial strains:		
P. aeruginosa UMTKB-5	Wild-type	This study
E. coli S17-1	RecA and tra genes of plasmids RP4 integrated into chromosome; auxotrophic for praline and thiamine	[15]
Plasmid:		
pBBR-PC1020	pBBR1MCS-2 derivative harboring approximately 1.7 kb fragment of PHA synthase gene from *Cupriavidus* sp. USMAA1020	[16]

2.2. Construction of *P. aeruginosa* UMTKB-5 transformant

Plasmid pBBR-PC1020 was introduced into wild-type *P. aeruginosa* UMTKB-5 as described in [17]. Successful transformation of the plasmid was confirmed by plasmid extraction and digestion using restriction enzymes. The plasmid was extracted using DNA-spinTM plasmid DNA purification kit (iNtRON Biotechnology, Korea) according to manufacturer's instruction and further digested with SalI and KpnI restriction enzymes. The presence of plasmid and successful construction of the transformant was confirmed by observing the gel electrophoresis (~7kb).

2.3. Biosynthesis of P (3HB) and RL

Cultivation was carried out in 250 ml Erlenmeyer flasks containing MSM medium, per liter: 2.80 KH_2PO_4, 3.32 Na_2HPO_4 and 0.5 NH_4Cl. Approximately 0.06 g/L (3% v/v) of preculture bacteria was transferred into 46 ml of MSM medium. The MSM medium was supplemented with hydrated $MgSO_4.7H_2O$ (0.25 g/L) and 50 µl of trace elements [18]. The cultures were incubated at 30 ºC, 200 rpm for 72 h. Cells were harvested by centrifuging at 4 ℃, 9000 rpm for 5 min. Supernatants were subjected to RL analysis. The cell pellets were stored in a deep freezer overnight and lyophilized by using freeze dried (Labconco Freeze Dry System / Freezone 4.5) for 72 h.

2.4. GC-FID analysis of P(3HB)

P(3HB) in lyophilized bacterial cell was transesterificated by acidic methanolysis in the presence of 15 % (v/v) sulfuric acid and 85% (v/v) methanol [19]. The gas chromatography analysis was performed using Gas chromatograph – Flame ionization detector (GC-FID) (Shimadzu, Japan) equipped with a SPB-1 capillary column (30 m, 0.25 mm, df 0.25 m) (Supelco). Synthetic air was used as detector gas and nitrogen gas was used a carrier gas. Methyl octanoate (Sigma, USA) was used as internal standard. A total of 2 µL samples was injected.

2.5. Orcinol assay

The orcinol assay was carried out to assess direct amount of rhamnolipid in the supernatant samples. Approximately 400 µl of culture supernatant will be extracted twice with 750 µl of diethyl ether. After being vortex for 3 min, the fractions were evaporated to dryness and 400 µl of pH 8 phosphate buffer was added. To 100 µl of each sample, 900 µl of orcinol reagent (0.19% of orcinol in 53 % v/v of H_2SO_4) was added. After heating for 30 min at 80 ℃, the samples were cooled at room temperature. The optical density of samples were determined using spectrophotometer, Varioskan™ Flash Multimode Reader (Thermo Scientific, USA) at 421 nm of wavelength [20].

3. Results and Discussion

3.1. Production of RL by wild-type *P. aeruginosa* UMTKB-5

Biosynthesis of P (3HB) and RL using various carbon sources at a fixed concentration of 20 g/L is shown in table 2. High concentrations of RL, above 250 mg/L was produced when glucose or sucrose was supplied. The wild-type strain was able to convert glycerol and glycerin pitch into RL with concentrations ranging from 122 to 149 mg/L when fed as carbon source. On the other hand, lower concentrations of RL were produced when molasses or sweet water was used. The wild-type strain was not able to produce any form of PHA. It is interesting to note that, high concentration of RL was produced from sucrose despite the low cell biomass. Low production of cell biomass indicates that the strain may lack the enzyme (i.e., invertase) for sucrose hydrolysis [21].

Table 2: Production of RL by wild-type *P. aeruginosa* UMTKB-5 using various carbon sources[a].

Carbon sources	Cell dry weight (g/L)	RL concentration[b] (mg/L)
Glucose	1.4 ± 0.2	272 ± 4
Fructose	1.3 ± 0.2	128 ± 4
Sucrose	0.5 ± 0.1	253 ± 7
Glycerol	1.5 ± 0.1	149 ± 9
Glycerin pitch	1.2 ± 0.1	122 ± 4
Molasses	2.5 ± 0.3	54 ± 1
Sweet water	0.6 ± 0.1	70 ± 2

Data shown are means of triplicates
[a]Incubated for 72 h at 30 ℃ at 200 rpm in MSM medium
[b]Concentration of RL as determined by orcinol assay

3.2. Production of RL and P(3HB) by transformant *P. aeruginosa* UMTKB-5

The transformant strain was able to produce both RL and P(3HB) in a single culture medium when supplied with different carbon sources with exception to molasses and sweet water. Compared to the wild-

type, in general there was a reduction in RL concentration produced by the transformant strain. The transformant was able to produce 43 to 57 mg/L of RL from the different carbon sources (Table 3). On the other hand, P(3HB) production was found to be in the range of 9 to 24 %wt. Highest P(3HB) content of 24 wt% was produced from glycerol. Reduced production of RL by the transformant when P(3HB) accumulation was initiated suggests that the some intermediate substrates used for these biomaterials are similar. The rh1G gene encoding a β-ketoacyl reductase is involved in the biosynthesis of rhamnolipids. The rhIG will catalyze the NADPH-dependent reduction of β-ketodecanoyl-ACP, which is intermediate of fatty acid de novo biosynthesis, thus resulting in β-hydroxydecanoyl-ACP, a putative precursor for biosynthesis of rhamnolipid. Both PHA and rhamnolipid contain lipid moieties which are derived from fatty acid biosynthesis. The proposed pathway for mcl-PHA and rhamnolipid biosynthesis suggested that both biosynthesis pathways are competitive. Therefore the intermediate substrates have to be simultaneously channelled for RL as well as P(3HB) production. The PHA synthase is the key enzyme for PHA biosynthesis [22]. The introduction of this gene into the wild-type strain has enable this bacterium to accumulate P(3HB). This shows that the strain has the ability to generate PHA intermediates through certain biochemical pathways but was missing the key enzyme of PHA polymerization.

The use of biosurfactant and bioplastics are generally restricted due to their relatively high production cost. This study was aimed to use by-products from oleochemical industry and sugar cane refinery for the production of P(3HB) and RL. The utilization of these cheap and renewable resources may help to reduce overall production cost. Wastes and by-products are considered as promising substrate for the production of biomaterials such as PHA and RL as it cheaper and can help overcome waste management problems [23].

Table 3: Production of RL and P(3HB) by transformant *P. aeruginosa* UMTKB-5 using various carbon sources[a].

Carbon sources	Cell dry weight (g/L)	RL concentration[b] (mg/L)	P(3HB) content[c] (wt%)
Glucose	1.6 ± 0.1	55 ± 1	13 ± 3
Fructose	2.0 ± 0.2	56 ± 5	17 ± 3
Sucrose	0.5 ± 0.1	55 ± 3	10 ± 1
Glycerol	2.1 ± 0.1	50 ± 8	24 ± 2
Glycerin pitch	1.4 ± 0.1	52 ± 5	18 ± 1
Molasses	3.0 ± 0.1	43 ± 2	N.D
Sweet water	0.4 ± 0.1	57 ± 1	N.D

P(3HB), poly(3-hydroxybutyrate)
Data shown are means of triplicates.
[a]Incubated for 72 h at 30 ℃ at 200 rpm in MSM medium
[b]Concentration of RL as determined by by orcinol assay
[c]P(3HB) content in freeze-dried cells were determined using GC-FID
N.D = Not detected

4. Conclusion

The transformant *P. aeruginosa* used in this study was able to simultaneously produced P(3HB) and RL using carbon sources such as glucose, fructose, sucrose, glycerol and glycerine pitch in a single culture medium. Molasses and sweet water were successfully converted into RL by both wild-type and transformant strains. Bioconversion of by-products from oleochemical industry and sugar cane refinery for the production of these value-added biomaterials is indeed a good way to reduce carbon substrate cost in the fermentation process as well as to overcome waste management issues by the industries. The production of RL and P(3HB) from these renewable materials can be further optimized in future studies.

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