Effect of carbon and nitrogen sources on simultaneous production of α -amylase and green food packaging polymer by *Bacillus* sp. CFR 67

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Abstract In this paper, effect of different carbon and nitrogen sources, including hydrolysates of rice bran and wheat bran, on simultaneous production of α -amylase (for hydrolysis of starch in food systems) and polyhydroxyalkanoates (PHA, a green biopolymer, which can be used as a packing material for foods) by *Bacillus* sp. CFR 67 was studied by submerged fermentation. Amongst various carbon sources tested, glucose and sucrose supported production of significantly ($P<0.05$) higher amount of α -amylase (66 U/ml) and PHA (444 mg/l), respectively. Of the nitrogen sources tested, ammonium acetate and beef extract led to the production of maximum amount of amylase (36 U/ml) and PHA (592 mg/l), respectively. Supplementation of the production medium with wheat bran hydrolysate (50 ml/l) produced significantly higher amounts of amylase (73 U/ml) and PHA (524 mg/l). Thus this study indicated the potential of agro-residues for the production of value added biomolecules, which can reduce the cost of production of these molecules and enables to reduce the pollution mainly caused by the use of non biodegradable plastics.

Keywords Amylase · *Bacillus* · Carbon · Nitrogen · Polyhydroxyalkanoates · Rice bran hydrolysate · Submerged fermentation · Wheat bran hydrolysate

Introduction

Microbial amylases are in great demand in various sectors such as pharmaceuticals, food, textile and detergents. Both

α - and β -amylases find applications in food processing, such as baking, brewing and distillery industries. Polyhydroxyalkanoates (PHA), a type of biodegradable polyesters, also known as green bioplastics, are intracellular carbon reserve materials produced by bacteria. Properties of PHA are comparable to polypropylene, thus the non biodegradable conventional plastics can be replaced with PHA for food packaging applications (Siracusa et al. 2008). Additionally, PHA are also used in agriculture, pharmaceutical and other industries. Several bacterial species belonging to the genera *Bacillus* are known to produce amylases (Dhanya et al. 2006; de Carvalho et al. 2008; Hashemi et al. 2010; Joshi 2011; Ray et al. 2008; Uysal et al. 2011) and PHA (Halami 2008; Shamala et al. 2003; Vijayendra et al. 2007). Recently, Saranya Devi et al. (2012) reported simultaneous production of PHA and exopolysaccharides by *Sinorhizobium meliloti*. However, no reports are available on simultaneous production of amylases and PHA by *Bacillus* sp.

The major drawback for the use of PHA is its high production cost. Hence, it could not replace conventional plastics that are derived from petroleum products completely. In economics of PHA production, the cost of carbon substrate alone accounts for 50% of the total PHA production (Kim 2000). As a result, it is relevant to exploit cheap carbon sources to enhance the PHA content and productivity. Therefore, less expensive carbon sources such as date syrup (Omar et al. 2001), starch (Huang et al. 2006, Halami 2008), mahuva flower extract (Anil kumar et al. 2007), corn steep liquor (Vijayendra et al. 2007), biowaste (Kumar et al. 2009), jackfruit seed hydrolysate (Ramadas et al. 2010) have been used as a carbon source for PHA production by *Bacillus* spp. Similarly, wheat bran and groundnut oil cake (Gangadharan et al. 2008), wheat bran and sunflower oil cake (Rajagopalan and Krishnan 2010), sugarcane bagasse

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hydrolysate (Rajagopalan and Krishnan 2008) and wheat bran by solid substrate fermentation (Hashemi et al. 2010) have been used for the production of α -amylase by *Bacillus*. The present work was carried out to evaluate the effect of different carbon sources including several starches, nitrogen sources and hydrolysates of wheat bran and rice bran for simultaneous production of α -amylase and PHA by *Bacillus* sp. CFR 67.

Materials and methods

Inoculum preparation *Bacillus* sp. CFR 67 obtained from the culture collection of department of Food Microbiology, CFTRI, Mysore, was maintained on nutrient agar slants at 4 °C. Before use, it was subcultured twice using nutrient broth. Inoculum was prepared by transferring 100 μ l of the culture into 10 ml of sterile nutrient broth and incubating at 30 °C for 18 h on a rotary shaker (200 rpm). The inoculum with 3×10^6 cfu/ml was used in all the experiments at 10% (v/v) level.

Effect of different carbon sources on PHA and α -amylase production The production medium containing (g/l) $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ –2.2, KH_2PO_4 –1.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ –0.2, $(\text{NH}_4)_2 \text{SO}_4$ –1.5 (0.32 g N/l), was supplemented with different carbon sources like, corn starch, glucose, sucrose, potato starch, tapioca starch, and soluble starch, individually at 20 g/l concentration. The initial pH was adjusted to 7.0, dispensed into 250 ml Erlenmeyer flasks (50 ml/flask) and was sterilized. The medium flasks were inoculated and incubated at 30 °C on a rotary shaker at 200 rpm for 72 h.

Effect of different nitrogen sources on PHA and α -amylase production The PHA production medium described above was prepared using different organic and inorganic nitrogen sources, as listed in Table 1. Sucrose at 20 g/l was used as a carbon source. The nitrogen sources were added to the medium individually at a concentration of 0.2 g N/l. The medium was sterilized, inoculated and incubated as mentioned above.

Effect of nutrient supplements on PHA and α -amylase production One hundred grams of wheat bran and rice bran were suspended individually in 1 l of water and pH was adjusted to 7.0. To this, α -amylase (5000 U) (Anil starch industries, Ahmedabad) was added and kept at 80 °C for 30 min and cooled. After adjusting the pH to 5.5, using dilute acid, amyloglucosidase (6000 U) (Anil starch industries, Ahmedabad) was added and incubated at 45 °C for 5 h. The hydrolysates were filtered and the volume was made up to 1 l. This was used directly at various levels in the medium.

The production medium prepared using sucrose (20 g/l) as a carbon source was supplemented with hydrolysates of rice bran and wheat bran at different concentrations (10, 25 and 50 ml/l), either individually or in combination. The initial pH was adjusted to 7.0. The medium was sterilized, inoculated with the culture at 10% (v/v) level and incubated at 30 °C on a rotary shaker at 200 rpm for 72 h.

Analysis of fermented broth The cells were separated from the broth by centrifugation at 8000 rpm for 15 min, washed twice using saline and dried to a constant weight at 90 °C and the dry weight was expressed as g/l. The dried cells were hydrolysed using sodium hypochlorite and the PHA was extracted using chloroform as described earlier (Shamala et al. 2003). The α -amylase content in cell free supernatant was determined using the reported method (Manning and Campbell, 1961). The assay was carried out at 60 °C for 10 min and enzyme activity (U) was defined as mg of starch hydrolysed per minute under the assay conditions.

Statistical analysis All the experiments were conducted twice in duplicate and standard deviation was determined. To determine the significance, the data was analysed by one way anova using Microsoft excel software 2003.

Results and discussion

Effect of different carbon sources on α -amylase and PHA production Data in Table 1 indicated that the growth of *Bacillus* sp. CFR 67 and PHA production vary depending upon the carbon source used in the medium. The maximum dry biomass content was observed with corn starch; however, the highest PHA yield (38.4%) was noticed with sucrose. Among the carbon sources tested, the medium prepared with glucose resulted in significantly ($P < 0.05$) higher amylase yield. Contrary to this, Thippeswamy et al. (2006) have noticed 4 times increased production of amylase with maltose than with glucose and Sudharhsan et al. (2007) have reported production of highest amount of amylase (31 U/ml) by a *Bacillus* sp. with lactose as a carbon source. They have observed repression effect of glucose and fructose on amylase production. Even though higher amounts of PHA and amylase were produced by sucrose and glucose, respectively, the cost of these carbon sources is high when compared to the other carbon sources such as starches.

Negi and Banerjee (2010) have opined that not all carbon and nitrogen sources would act as enhancers for the production of amylase and proteases. In the present study, *Bacillus* produced more of biomass rather than PHA with corn starch. Of the starches used, the tapioca starch has given maximum yield of PHA (29.1%) and soluble starch supported production of significantly ($P < 0.05$) higher yields of α -amylase.

Table 1 Effect of carbon and nitrogen sources on production of polyhydroxyalkanoates (PHA) and amylase by *Bacillus* sp. CFR 67

Nutrient	Dry biomass ^a (g/l)	PHA (mg/l)	Amylase (U/ml)
a) Carbon source			
Corn starch	3.2±0.59 ^a	94±1.25 ^a	51.7±1.28 ^a
Soluble starch	2.0±0.21 ^b	378±5.23 ^b	56.6±1.42 ^b
Tapioca starch	1.3±0.12 ^c	390±6.25 ^c	35.0±1.26 ^c
Potato starch	1.4±0.18 ^c	242±2.35 ^d	17.8±0.98 ^d
Glucose	1.3±0.16 ^c	444±8.55 ^e	62.4±1.52 ^e
Sucrose	1.2±0.12 ^c	480±5.88 ^f	40.6±1.46 ^f
b) Inorganic nitrogen source			
Sodium nitrate	2.9±0.22 ^a	286±4.52 ^a	14.4±1.28
Potassium nitrate	2.0±0.24 ^b	200±2.46 ^b	ND
Diammonium hydrogen-orthophosphate	3.8±0.34 ^c	4±0.32 ^c	35.0±2.12 ^a
Ammonium chloride	2.3±0.28 ^b	168±2.48 ^d	31.1±2.00 ^b
Ammonium sulphate	2.2±0.26 ^b	200±2.02 ^e	17.7±1.02 ^c
Ammonium nitrate	3.0±0.32 ^a	8±0.62 ^f	ND
Ammonium acetate	1.8±0.18 ^d	192±2.48 ^g	36.6±2.24 ^d
c) Organic nitrogen source			
Glycine	2.1±0.22 ^a	36±1.02 ^a	ND
Peptone	3.5±0.38 ^b	40±0.80 ^b	ND
Beef extract	3.2±0.32 ^b	592±5.26 ^c	17.7±1.24 ^a
Yeast extract	3.2±0.36 ^b	394±3.2n	21.1±1.12 ^b
Tryptone	2.4±0.26 ^c	12±0.68 ^e	ND
Urea	3.4±0.32 ^b	200±3.42 ^f	ND

^aBiomass weight includes the weight of PHA; ND: not detected. In the experiment on different carbon sources, ammonium acetate was used as a nitrogen source. In the experiment on different nitrogen sources, sucrose was used as a carbon source. Results are means of 2 trials done in duplicate±standard deviation. Different superscript letters indicate significant difference within the same column at $P<0.05$

Fig. 1 Effect of bran hydrolysates on production of α -amylase and polyhydroxyalkanoates (PHA) by *Bacillus* sp. CFR 67. RBH rice bran hydrolysate; WBH wheat bran hydrolysate

Similar to our observation, *Bacillus cereus* SPV was found to produce PHB at a concentration of 38% of its dry cell weight in shaken flask cultures, using glucose as a main carbon source (Valappil et al. 2007) and *B. mirini* showed the maximum enzyme activity in the presence of starch than the monosaccharides tested (Ashwini et al. 2011). However, maximum production of PHA by a pigment producing *Micrococcus* sp. using sucrose was reported earlier (Vijayendra et al. 2008), indicating that the ability to use the carbon source to produce PHA varies with the type of the organism. These variations may be due to the change in carbon source, strain variation or growth conditions.

Effect of different nitrogen sources on PHA and α -amylase production Both organic and inorganic nitrogen sources were tried to optimize the source of nitrogen for PHA and amylase production. Sodium nitrate, an inorganic nitrogen source, significantly ($P<0.05$) supported for the production of higher amount of PHA, but the yield of α -amylase was not significant (Table 1). Ammonium acetate supported production of both amylase and PHA and it is known to enhance PHA synthesis by *B. megaterium* (Gouda et al. 2001). Among the organic nitrogen sources tested, beef extract significantly ($P<0.05$) supported higher synthesis of PHA, however, amylase production was not significant. Contrary to this, yeast extract resulted in the production of maximum amount of amylase and a reasonable quantity of PHA. Tryptone and peptone were found to support production of biomass only. Organic sources of nitrogen resulted in production of higher amount of PHA than inorganic sources of nitrogen. Our results confirm an earlier observation that complex nitrogen sources like peptone, tryptone, yeast extract, soybean meal and beef extract results in production of higher amount of thermostable amylase than simple inorganic sources of nitrogen like various salts of ammonia and potassium (Thippeswamy et al. 2006). A complex nitrogen source appears to be essential for the production of higher amounts of amylase, which may be explained by the organism's preference for protein as a nitrogen source for growth rather than for extracellular enzyme formation.

Effect of bran hydrolysates on PHA and amylase production Brans of wheat or rice are rich sources of proteins and minerals. Sudha et al. (2011) have reported presence of 16–17% protein in rice bran along with good amount of minerals. Different concentrations (1, 2.5 and 5%) of RBH and WBH, either individually or in combination were used to culture *Bacillus* sp. CFR 67. The biomass content increased from 1.26 g/l to 3.21 g/l with enhanced supplementation of RBH content from 10 to 50 ml/l. However, maximum PHA content was obtained with 25 ml/l RBH concentration (Fig. 1) and maximum amylase was produced with 10 ml/l of RBH

concentration. Similar to RBH, with increasing concentration of WBH from 10 to 50 ml/l, there was an increase in biomass content from 0.52 g/l to 2.17 g/l. Similarly, amylase and PHA yields increased with the increase in the concentration of the WBH supplement in the medium. The reason for not getting large quantity of PHA at higher concentration of bran hydrolysates may be due the presence of more amount of protein, as in general it is that PHA are produced with excess carbon and low nitrogen content in the medium. However, the amount of PHA produced with WBH supplementation was more than double when compared to the PHA produced with RBH supplementation. In the present study, approximately 20% increase in amylase content was noticed with the supplementation of WBH than with RBH. Similarly, Sudharhsan et al. (2007) revealed maximum yield of amylase by *Bacillus* sp. in wheat (27 U/ml), which may be due to its higher starch content. Among the growth media containing various combinations of agro-residues, the medium with wheat bran and sunflower oil cake supported highest enzyme production (20,700 IU/g dry wt.) in solid substrate fermentation by *B. subtilis* KCC103 (Rajagopalan and Krishnan 2010). In the present study, when RBH and WBH were used in combination, the results were not that encouraging and the amylase yield dropped to 45.6 U/ml, which may be due to the inhibitory activity of these substrates when used in combination.

In conclusion, *Bacillus* sp. CFR 67 can be exploited for simultaneous production of amylase and PHA using agro industry residues as a substrate, which can reduce the production cost besides reducing the disposal problem of these substrates. Optimization of production parameters can further boost the yields of these molecules.

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