Exploration of rice bran, an agro-industry residue, for the production of intra and extra cellular polymers by *Sinorhizobium meliloti* MTCC 100

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Abstract

The present work was focused on simultaneous production of certain intra and extra cellular polymers by fast growing *Sinorhizobium meliloti* MTCC 100 using rice bran, a low cost agro-industry residue, in hydrolysed form to enhance product yields. The culture produced 3.63 g/L of biomass, 1.75 g/L of intra cellular polymer (polyhydroxyalkanoate, PHA) and 1.2 g/L of extra cellular polymer (exopolysaccharide, EPS) in control, polymer production (PP) medium. Supplementation of 20% rice bran hydrolysate (RBH) to PP medium at 0 h resulted in increased production of biomass, PHA and EPS (5.92, 2.71 and 2.01 g/L, respectively). Addition of RBH at after 24 h of fermentation increased the amount of EPS by 5 folds after 72 h at 30°C. An initial pH of 7.0 and fermentation temperature of 30°C were found to be optimum for the production of biomass as well as both the biopolymers. The biomass, PHA and EPS contents increased with the increase in fermentation period from 24 h to 72 h, with a maximum biomass of 7.45 g/L and PHA of 3.60 g/L. With further incubation to 96 h EPS production increased to 11.8 g/L. Gas chromatography and Fourier transform infra red spectroscopy of the PHA indicated it to be a copolymer of polyhydroxybutyrate and polyhydroxyvalerate.

Keywords: characterization, exopolysacchride, polyhydroxyalkanoate, rice bran hydrolysate, *Sinorhizobium meliloti*,

Introduction

Accumulation of non biodegradable petrochemical derived plastic waste in the environment is increasing day by day and posing a great threat to the global environment. For this reason, researchers all over the world are working on production of biodegradable plastics, such as polyhydroxyalkanoates (PHA), which are produced as intra cellular polymer, by many microorganisms. The PHA extracted from bacterial cells show material properties that are similar to polypropylene [1]. Accumulation of PHA is a natural way for bacteria to store carbon and energy when nutrient supplies are imbalanced. These polyesters are accumulated when bacterial growth is limited by depletion of nitrogen, phosphorus or oxygen and presence of excess amount of a carbon source [2-3].

Extra cellular polymers (also known as exopolysaccharides, EPS) protect bacterial cells from unfavourable environment and provide the energy and carbon source when the substrate is in short supply. EPS are commercially useful for producing gels and modifying the rheological properties of aqueous systems and foods. They have potential in replacing plant and algal EPS that are traditionally being used in food, pharmaceutical, textile and oil industries. Production of EPS by *Rhizobium* spp. is essential for nodule invasion; however, their exact role in symbiosis is unknown [4]. Biosynthesis of exopolysaccharides in *Rhizobium* spp. is a very complex process regulated at both transcriptional and post translational levels and influenced by various environmental conditions [5].

Currently the cost of the PHA and EPS is a major cause which is preventing their use in large scale. The cost of the carbon accounts for more than 50% of the biopolymer production cost. Hence, all over the world researchers are trying to use various carbon sources, including agro-industry residues such as plant oil cakes (palm oil, coconut oil, cotton seed, ground nut oil,

soybean oil, sunflower oil cake etc.), rice bran oil, sugar cane baggase, molasses, corn steep liquor, triacylglycerols (vegetable oils and animal fats), cheese whey, meat bone meal, sludge, waste water etc [6-13] for the production of PHA. Use of industrial waste as a fermentation substrate for the production of *Rhizobium* spp. biomass is in practice [14].

Fast growing rhizobia are able to synthesize a high molecular weight EPS [15]. *Rhizobium* spp. are known to accumulate up to 58% PHA of the biomass [16] and mutation of *Rhizobium meliloti* increased the production of PHA from 57% to 69% [17].

Although many studies have been carried out on production of PHA [8, 11, 17-19] or EPS [20-23] by *Rhizobium* spp., very few reports are available on simultaneous production of intra cellular (PHA) and extra cellular (EPS) polymer production by *R. meliloti* [24-25]. The aim of the present work was to produce both intra (copolymer of polyhydroxybutyrate-co-polyhydroxyvalerate, [P(HB-co-HV)]) and extra cellular biopolymers simultaneously from a fast growing *Sinorhizobium meliloti* (formerly known as *R. meliloti*) MTCC100 using rice bran hydrolysate (RBH) as a supplement and this is the first report on simultaneous production of both the polymers using rice bran, an agro-industry residue, as a supplement.

Materials and methods

Microorganism and inoculum preparation

Sinorhizobium meliloti MTCC 100 (Institute of Microbial Technology, Chandigarh, India) was used in the study for the production of biopolymers. The strain was maintained on *Rhizobium* medium (Hi-media Laboratories Ltd., Mumbai, India) slants and stored at 4°C for further use. It was subcultured twice before preparing the inoculum. The inoculum was prepared by transferring entire culture from a slant into 50 ml of the inoculum medium (composition, g/L): Na_2HPO_4 - 4.4, KH_2PO_4 -1.5, $(NH_4)_2\ SO_4$-1.0, $MgSO_4 \cdot 7\ H_2O$-0.2, sucrose-10.0 and yeast

extract-0.5, pH-7.0) in 250 ml Erlenmeyer flask and incubated on a rotary shaker (200 rpm) at 30°C for 24 h.

Preparation of rice bran hydrolysate

Fresh rice bran was obtained from a local rice mill. The substrate (100 g) was suspended in one liter of distilled water and the starch was hydrolysed by α-amylase (5000 U, Anilozyme, Anil starch Industries, Ahmadabad, India) at 80°C for 30 min and amyloglucosidase (5000 U) at 50°C and pH 5.5 for 4 h and cooled to 40°C. Protein was hydrolysed using alcalase 2L (51 U, Novozyme) and lipids by lipase (1300 U, Zeus Biotech, Mysore, India) for 1 h. The RBH was filtered and centrifuged (6000 rpm for 15 min) to remove suspended particles and volume was made up to one liter.

Optimization of fermentation conditions

To determine the effect of supplementation of RBH, the polymer production (PP) medium with 50 g/L of sucrose was used. Remaining components of the PP medium were similar to inoculum medium. It was distributed in 50 ml per 250 ml Erlenmeyer flask. The medium, when required, was supplemented either with 20% or 40 % (v/v) of sterilized (by autoclaving at 15 lbs for 20 min) RBH, either at initial stage or after 24 h of fermentation. The PP medium without added RBH served as a control. After adding 10% (v/v) of inoculum (with a viable count of 2.2×10^8 /ml), the flasks were incubated on a rotary shaker (200 rpm) at 30°C for 72 h.

To know the effect of initial pH on production of PHA and EPS, the initial pH of the PP medium supplemented with 20% RBH was adjusted to 6.0, 7.0 and 8.0, individually and autoclaved as mentioned earlier. The flasks were inoculated with 10% (v/v) of culture, incubated at 30°C on a rotary shaker (200 rpm) for 72 h.

126 To determine the effect of temperature on PHA and EPS production, the PP medium
127 containing 20% RBH was prepared with an initial pH of 7.0 and autoclaved. After adding the
128 inoculum (10%, v/v), the medium flasks were incubated at different temperatures such as 30°C,
129 37°C and 40°C, individually, on rotary shakers (200 rpm) for 72 h.
130 Kinetics of PHA and EPS production by *S. meliloti* MTCC100 was studied using the PP
131 medium (pH 7.0) supplemented with 20% RBH. The sterilized medium flasks were inoculated
132 (10%, v/v) and incubated at 30°C on rotary shaker (200 rpm). The flasks were removed after 24,
133 48, 72 and 96 h. The fermented medium of all the experiments was analyzed for biomass, PHA,
134 EPS and residual sugar contents as mentioned in the following section.
135 *Analysis of fermented broth*
136 To determine the biomass content, the fermented medium was centrifuged at 8000 rpm for 20
137 min. The cell pellet was washed with distilled water and dried in hot air oven at 90°C till
138 constant weight and biomass weight (inclusive of PHA) was expressed in g/L. The PHA was
139 extracted from dried biomass using sodium hypochlorite method [26] and its weight was
140 expressed in g/L. The amount of PHA present in the biomass was calculated and expressed as %
141 PHA content.
142 EPS was harvested from the cell free supernatant by addition of 2 volumes of chilled
143 isopropyl alcohol. Precipitated EPS was collected and dried at 90°C till constant weight and the
144 content was expressed in g/L. The residual sugar present in the cell free supernatant was
145 estimated as reported earlier using dinitro salicylic acid method [27].
146 *Characterization of PHA*
147 To determine the composition, the PHA was extracted from the dried cells with chloroform at
148 40°C and the solubulized PHA was precipitated with hexane. It was subjected to methanolysis

and analysed by GC (Fissions 8000 series, Italy) using Varian Factor 4 capillary column (30 m x 0.25 mm, VF-1MS) and flame ionization detector. Nitrogen (1 ml /min) was used as a carrier gas [11]. PHA sample (3-4 mg) was mixed with 25 mg of IR grade KBr powder and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1} in FTIR Nicolet Magna 5700 spectrophotometer (Thermo electron Inc., NICOLET 5700, FT- Raman Module, USA).

Statistical analysis

All the experiments were conducted twice in duplicate and the data obtained were analysed by one-way ANOVA using Microsoft Excel 97 software.

Results and discussion

Effect of supplementation of RBH on polymer production

The rice bran is a rich source of nutrients, which can influence the growth of the bacteria or the quality of the polymer produced by it. The composition of rice bran as reported earlier [28] is (g%): moisture, 10; protein, 16; oil, 13; ash, 14; fiber, 14 and carbohydrates 27. The oil contained in rice bran was composed of (%): lauric acid, 0.4; palmitic acid, 21; oleic acid, 42; linoleic acid, 31; palmitoleic acid, 0.3; myristic acid, 0.4; stearic acid, 3; linolenic acid, 1 and arachidonic acid, 0.6. RBH prepared by hydrolysis of 100 g substrate in one liter of water contained 21g/L of soluble substrate, 8 g/L of free amino acids and 6 g/L of free fatty acids (unpublished data).

The PP medium was supplemented with RBH (at 20% or 40% level) either prior to incubation or after 24 h of incubation and the results are provided in Table-1. Although RBH enhanced the production of both intra cellular and extra cellular polymers by *S. meliloti* MTCC100, its effect was more pronounced in the production of EPS rather than PHA. Addition of RBH at initial stage of fermentation resulted in production of highest amount of biomass and the PP medium without RBH supplementation had lowest amount of biomass. However,

supplementation of RBH, either at 20 % or 40% level, after 24 h of fermentation reduced the quantity of both biomass and PHA. It is interesting to note that this late addition of RBH at both the concentrations increased the EPS production by 5 fold when compared to the yield obtained from PP medium (control) alone. Significant benefit of using 40% RBH over 20% RBH was not noticed, hence, further experiments were carried out using 20% RBH only. Enhanced EPS production may be due to the triggering of biosynthetic enzymes responsible for the production of EPS by the RBH, which is a rich nutrient supplement containing hydrolysates of starch, proteins and fats. Shamala et al. [11] reported production of a copolymer of short chain length PHA with sucrose and rice bran oil as carbon substrates by a local isolate of *R. meliloti*; however, supplementation of rice bran oil did not lead to significant increase in PHA content, but resulted in the production of copolymer of PHA. In contrast to the present findings, Huang et al [29] with a repeated fed batch fermentation, observed 3 fold increase in PHA production by *Haloferax mediterranei*, when the corn starch medium was supplemented with extruded rice barn (1:8 ratio).

Optimization of production of biopolymers

Various factors can affect the production of biopolymers by microorganisms. Microorganisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH optima, which are the critical factors for maximum EPS production [30]. The increase in yield and change in the quality of microbial EPS can be achieved by manipulating the culture conditions [31]. To increase the biopolymer productivity, optimization studies were carried out by determining effect of pH, temperature and time on simultaneous production of both intra and extra cellular biopolymers.

*Effect of initial pH***:** The pH is an important parameter which is known to influence microbial growth. The production of PHA and EPS was carried out at various initial pH such as 6.0, 7.0 and 8.0 and the results are presented in Fig.1. Highest growth and biomass production was obtained at pH 7 compared to that of pH 6 or pH 8. Although the amount of PHA produced at pH 8 was not significantly higher than at pH 7.0, the % PHA produced was slightly higher (54%) at pH 8 than at pH 7 (50.9%). Earlier studies revealed that mutation of *R. meliloti* lead to enhancement of PHA production from 57% to 69% when the fermentation was carried out at pH 7.0 [18]. In the present study, similar to PHA, EPS production by *S. meliloti* was slightly higher at pH 7.0 and 8.0 than at pH 6.0, with no significant difference in the amount of EPS produced either at pH 7.0 or pH 8.0. This may be due to the regulation of the biosynthetic pathway of EPS production, which may be dependent on pH [32].

Effect of temperature: Lower temperature (30°C) favoured growth and polymer production by *S. meliloti* compared to higher temperature (Fig. 2). This may be probably due to the reduced activity of the enzymes responsible for the synthesis of both the polymers or due to the low growth rate. Decrease in temperature causes a decrease in growth rate and cell wall polymer biosynthesis, producing more precursors available for EPS synthesis [29]. Therefore, Duta et al [22] have carried out the optimization studies for EPS production by *Rhizobium* sp. EQ1 at a constant temperature of 30°C and pH 7.0 and Tavernier et al [24] studied co-production of EPS and PHA by *R. meliloti* at similar conditions.

*Kinetics of dual polymer production***:** Time course analysis of PHA and EPS at optimized fermentation conditions of 30°C and pH 7 showed incremental enhancement in yields up to 72 h (Fig. 3). Thereafter, PHA content of the cell decreased, which may be due to activation of depolymerases and *in situ* utilization of the polymer by the bacterium beyond log phase of its

growth. This also resulted in the decrease of biomass weight, as the biomass weight includes the weight of the PHA. However, EPS production was favoured with prolonged fermentation, resulting in increased viscosity of the medium (data not shown), which may be due to complete depletion of nitrogen that supports further synthesis of EPS [33]. Literature reports indicate that nutrient depletion in the presence of surplus carbon is required for PHA production; however it is essential to define carbon concentration, otherwise excess carbon is diverted towards synthesis of biomass or other metabolites [17,18]. However, Tavernier et al [24] reported simultaneous production of very low amount of PHA (0.1-1 g/L) and EPS (0.1-1.2 g/L) by 2 different strains of *R. meliloti* using either glucose or fructose as a substrate in 2 different production media. They noticed limited growth in a nitrogen-deprived medium and it favored EPS production rather than PHB production. Similar to our findings, Ben Rebah et al [8] also observed a reduction in PHA content after 36 h when *S. meliloti* A_2 was grown in slaughter house waste or secondary sludge.

Characterization of PHA

Characterization of PHA by GC and FTIR indicated that the polymer produced in PP medium was only polyhydroxybutyrate and a copolymer of polyhydroxybutyrate-co-hydroxyvalerate [P(HB-co-HV)] of 98:2 in medium supplemented with RBH. This observation is in accordance with the report of Shamala et al [11], who have also reported the production of co-polymer by *R meliloti* with the addition of rice bran oil to the production medium. The FTIR spectrum of the co-polymer of PHA produced by *R. meliloti* (Fig. 4) indicated peaks at 1725 attributing to the stretching vibration of the C=O group (ethyl carbonyl) in the polyester. Bands at 1183, 1227 and 1279 cm^{-1} indicated presence of C-O-C group. Transmission bands at 2877, 2934 and 2977 were attributed to stretching vibration of CH bonds of methyl (CH_3) and methylene (CH_2) group. Other characteristic bands for scl-PHA were noticed at 2997, 1100, 1057 (C-O), 979 and 515

242 cm^{-1}. Similar observations were made by Shamala et al [11], indicating that the polymer
243 produced is a co-polymer of [P(HB-co-HV)].

244 **Conclusions**

245 This is the first report detailing the simultaneous production of intra and exocellular polymers by
246 *Sinorhizobium meliloti* using RBH as a supplement. Further, RBH supplementation enhanced the
247 production of both the polymers (3.6 and 11.8 g/L of PHA and EPS, respectively), besides
248 producing a maximum of 7.45 g/L of biomass. The increased production of polymers using low
249 cost nutritional supplement can reduce the production cost of these polymers. Production of
250 value added biopolymers, especially the co-polymer of PHA can reduce the use of petroleum
251 derived non biodegradable plastics and thereby reducing the environmental pollution. The EPS
252 produced also has a potential for industrial applications.

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254
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258 **Conflict of Interest:**

259 All authors declare that there is no conflict of interest including any financial, personal or other
260 relationships with other people or organizations.

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262
263 **Author's disclosure:**
264
265 Ms. Saranya Devi carried out the research the work. Dr. Shamala has planned the work and Dr.
266 Vijayendra has supervised the work and prepared the manuscript. All authors have approved the
267 final article.

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Legends to figures:

Fig.1- Effect of initial pH of rice bran hydrolysate supplemented medium on intra and extra cellular polymer production by *S. meliloti* MTCC 100 at 30°C and 200 rpm

Fig. 2- Effect of temperature on intra and extra cellular polymer production by *S. meliloti* MTCC 100 in rice bran hydrolysate supplemented medium at pH 7.0 and 200 rpm

Fig.3 – Kinetics of intra and extra cellular polymer production by *S. meliloti* MTCC 100 in rice bran hydrolysate supplemented medium (pH-7) at 30°C and 200 rpm

Fig.4. FTIR spectrum of polyhydroxyalkanoates produced by *R. meliloti* MTCC 100 in rice bran hydrolysate supplemented medium

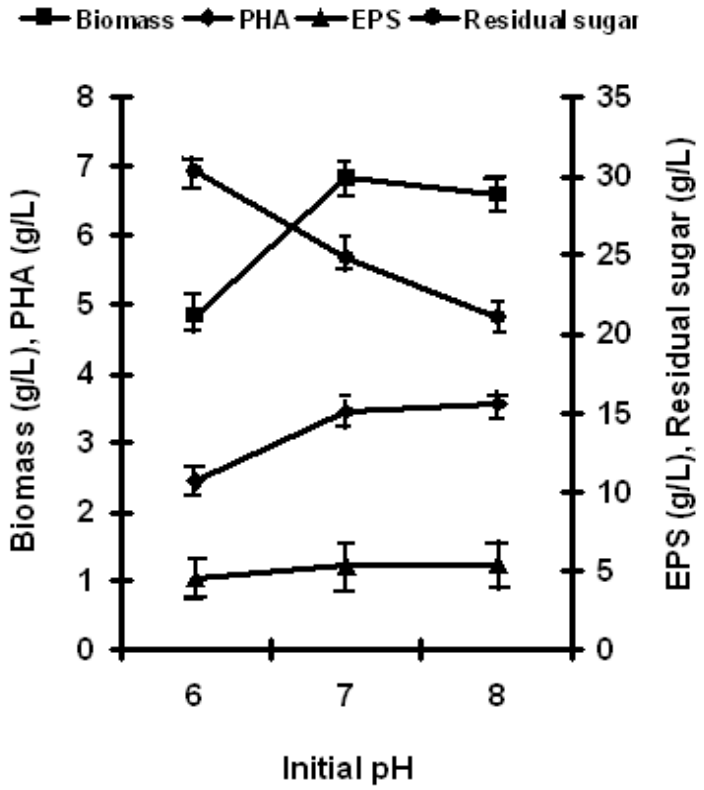
Table 1

Effect of supplementation of rice bran hydrolysate (RBH) on intra and extra cellular polymer production by *Sinorhizobium meliloti* MTCC100

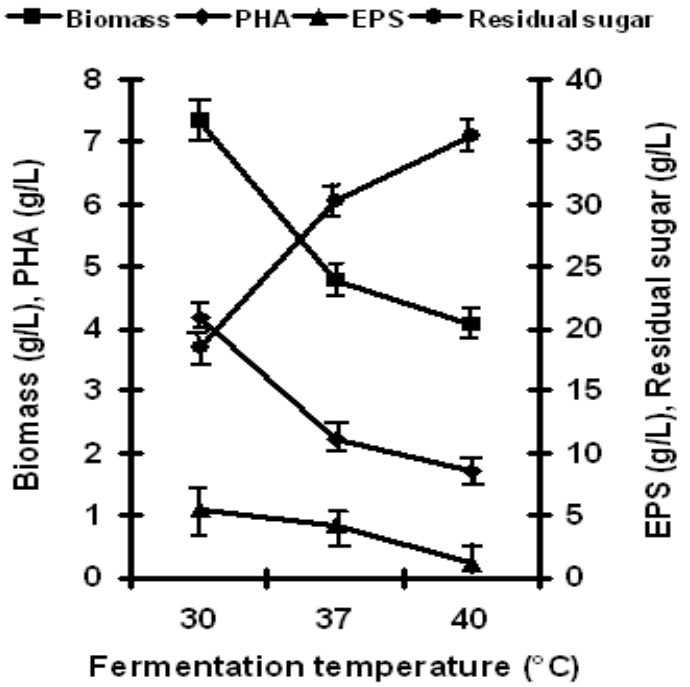
Medium	Analysis parameters			
	Biomass (g/L)	**PHA (g/L)**	**EPS (g/L)**	**Residual sugar (g/L)**
PP medium (control)	3.63±0.4[a]	1.75±0.3[a]	1.20±0.2[a]	26.24±4.0[a]
PP medium + 20% RBH at 0 h	5.92±0.5[b]	2.71±0.2[b]	2.01±0.4[b]	24.65±6.0[b]
PP medium + 40% RBH at 0 h	5.40±0.5[b]	2.67±0.6[a]	2.67±0.4[b]	20.30±3.0[c]
PP medium + 20%RBH at 24 h	4.29±0.6[c]	1.64±0.2[a]	6.18±0.6[c]	27.52±4.0[a]
PP medium + 40%RBH at 24 h	3.27±0.3[a]	1.00±0.2[a]	6.45±0.5[c]	29.20±4.0[d]

PP medium: polymer production medium, PHA: polyhydroxyalkanoates; EPS: exopolysaccharide; Analysis was carried out after 72 of fermentation at 30°C on a rotary shaker (200 rpm). Results are average of two experiments done in duplicate ± standard deviation. Means without a common lower case letter within a column are significantly different ($p < 0.05$).

415 Fig.1



416
417 Fig.2
418



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422 Fig.3
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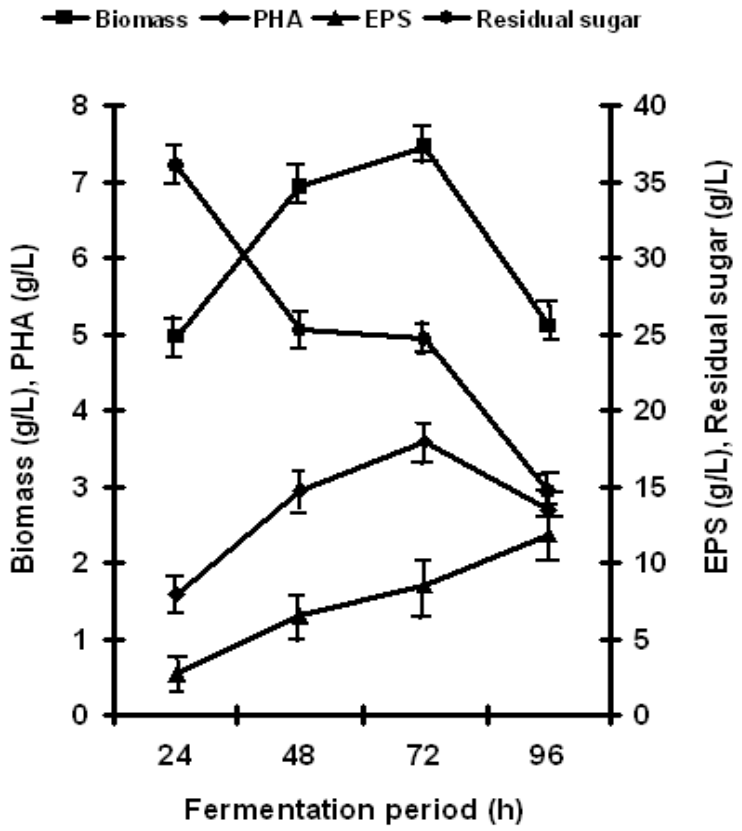
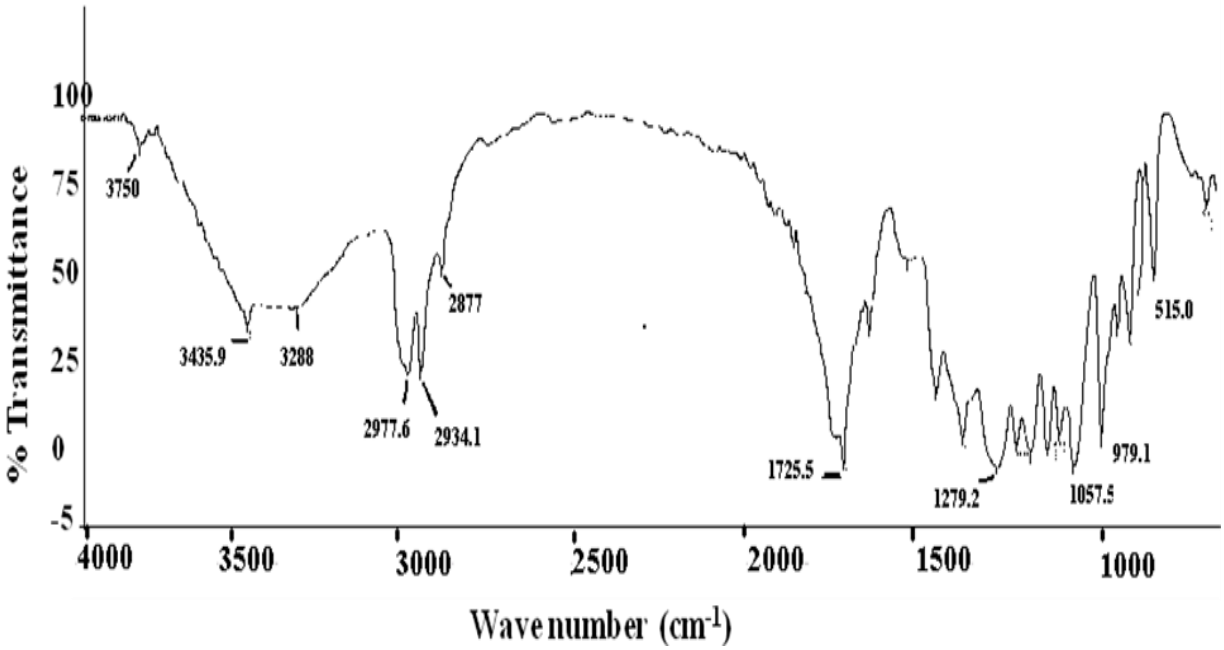


424
425 Fig.4



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