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Exobiopolymer from polyhydroxyalkanoate-producing transgenic yeast

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Recently, the wild type yeast *Kloeckera* sp. strain KY1 was equipped in their cytoplasm with the phaABC operon containing genes phbA, phbB and phbC of the PHA biosynthetic pathway of *Ralstonia eutropha*. Unpredicted, resulted transgenic yeast strain KY1/PHA was able to synthesize another exopolymer beside the production of PHA. Subsequently, produced exopolymer was subject for further identification, characterization and analysis. Partial purification of exopolymer was performed and characterized as glycoprotein. HPLC analysis of the polymer revealed the presence of a fructose chain. The functional group analysis by FT-IR spectroscopy showed the presence of carboxyl, hydroxyl and amid groups. The exopolymer was soluble in water and insoluble in any tested organic solvents and could flocculate kaolin suspension (5 g/l) over a wide range of pH (pH 3 to 9) and temperature (5 to 50°C) tested in the presence of $CaCl_2$. The highest flocculation activity of 99% for kaolin suspension was achieved at a dosage of 13 mg/l. Thus, it is possible that this glycoprotein could be substituted for a commercial polymer with respect to flocculation.

Key word: Transgenic yeast, bioflocculant, exopolymer, glycoprotein, spectroscopic analysis.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are structurally simple macromolecules synthesized by many Gram-positive and Gram negative bacteria. They accumulated as discrete granules to levels as high as 90% of the cell dry weight as intracellular storage materials which can be accu-mulated by a wide variety of microorganisms (Madison and Huisman, 1999). PHAs are thermoplastic and biodegradable polyesters, which can be used in the same way as many conventional petrochemical plastics (Breuer and Bma, 1999). However, the high price of the production of PHAs in prokaryotic system compared to synthetic non biodegradable polymers still limits their applications. Therefore, with the aim of reducing the production costs, yeast cells were used as models to gain information on PHAs synthesis. Engineering of novel pathways in eukaryotic cell systems seems to be a beneficial alternative to the production of PHAs in bacteria. Yeast cells can be used as models to gain information on PHAs synthesis in eukaryotes. In addition, yeasts as hosts for synthesis of PHAs have certain advantages over bacteria. Firstly, yeasts have been intensively studied from physiology, molecular biology and biotechnology points of views. Secondly, yeasts are physiologically flexible and they are larger than bacteria (Terentiev et al., 2004). Previously, the wild type yeast *kloeckera* sp. strain KY1/PHA was equipped in their cytoplasm with the phaABC operon containing genes phbA, phbB and phbC of the PHA biosynthetic pathway of *Ralstonia eutropha* (Abd-El-Haleem et al., 2007). Under optimum cultivation condition, transgenic yeast strain KY1/PHA was able to produce ~ 7% PHA of its cell

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Abbreviation: PHAs, Polyhydroxyalkanoates.

dry weight. However, beside PHA production, it was able to synthesize another extracellular polymer in high amount. Subsequently, the exopolymer was isolated, characterized and subjected for further bioflocculation experiments.

It is established that in comparison with the widely used chemical flocculants, including aluminum sulfate, ferric chloride, and polyacrylamide that have been widely used, bioflocculants show more advantages because of their safety and biodegradability (Xiong et al., 2010; Crescenzi, 1995; Kurane et al., 1994). Screening for novel microorganisms which could produce bioflocculants with excellent flocculating activity, cost-effective production process and high polymer yield is one of the main targets of many research groups worldwide (Zhang et al., 2002; Deng et al., 2003; He et al., 2004; Zheng et al., 2008). Therefore, this study was aimed to benefit from the unplanned exopolymer produced from the genetically modified yeast strain KY1/PHA which produces PHA as bioflocculant. Flocculation activity, isolation, purification and characteri-zation of the extracellular biopolymer were investigated.

MATERIALS AND METHODS

Identification of bioflocculant-producing transgenic yeast

As a first indicator of bioflocculant production and to ensure that transgenic yeast strain KY1/PHA has the ability to form slime and mucoid colonies, yeast cells were cultivated on YPD agar plate for 24 h at 30°C and then tested using toothpick (Abd-El-Haleem et al., 2008). Subsequently, the transgenic yeast was cultivated on basal medium (50 ml) containing (w/v) 1% glucose, 0.35% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4$ $7H_2O$ and 0.01% NaCl on a rotary shaker (200 rpm) at 30°C for 3 days. At the end of cultivation, the culture broth was measured for flocculating activity. Previously, strain KY1/PHA was identified by Abd-El-Haleem et al. (2007) as *Kloeckera* sp. using the API20C Aux system (BioMerieux Vitek, Inc., Hazelwood, Mo.). According to the method described by Bozzola and Russell (1999), yeast cells were subjected for microscopic characterization using scanning electron microscope (SEM) (JSM-6400, JEOL Ltd., Japan).

Determination of flocculating activity

Bioflocculating activity was determined with jar test equipment (Jar Tester Model CZ150) comprising six paddle rotors (24.5 and 63.5 mm), equipped with 6 beakers of 1 L each. The mixture containing 190 ml kaolin clay suspension (5 g/l, pH 7.0), 0.5 ml sample and 10 ml $CaCl_2$ solution (1%, pH 7.0). Subsequently, the mixture was stirred with rapid mixing at 200 rpm for 2 min, followed by slow mixing at 80 rpm for 3 min and left standing for 3 min. The supernatant was measured for absorbance at 550 nm. A control was prepared using the same method but the sample was replaced by distilled water. The flocculating activity was calculated according to the equation:

Flocculation activity = A550c-A550s/A550c * 100,

Where, A550s is the absorbance of the sample and A550c is the absorbance of the control.

Time course of bioflocculant production

The strain was cultivated in basal medium (pH 7.0). One loop of the 24 h culture was inoculated into 400 ml of the production medium contained in a 1000-ml flask and cultivated on a rotary shaker at 200 rpm, 30°C for 7 days. Samples were taken every 12 h to measure the optical density (OD 600 nm) and flocculating activity (Dermlmt et al., 1999; Wu and Ye, 2007).

### Partial purification of the bioflocculants

To partially purify the bioflocculant, culture broth of the strain was centrifuged to remove cells by centrifugal separation (4000 g, 30 min). Subsequently, cell-free supernatant was freeze-dried. The lyophilized precipitate was redissolved in an appropriate amount of distilled water and mixed with four volumes of cold 95% ethanol and left for 4 h at 4°C. After centrifugation at 7500 g, 4°C for 20 min, the aforementioned step was repeated for two additional times and the final precipitate was dried.

### Characterization of the partially purified biopolymers

An infrared spectrum (IR) of the exopolymer was measured on a KBr disk with a Perkin-Elmer series 1600 FT-IR to determine the functional groups of the biopolymer. The protein content was determined according to Bradford method (Bradford, 1976). Total sugars were determined by the phenol-sulfuric acid method (Chaplin and Kennedy, 1994). Additional analysis including the solubility test in distilled water and several solvents such as acetone, carbon tetrachloride, ethanol, isopropanol, hexane, methanol and nitrobenzene (Collins et al., 1973) was performed. The effect of pH (3 to 12) and temperature (5 to 100°C) on the flocculation activity was studied by measuring the flocculation activity of the reaction mixture containing the optimum concentration of exopolymer at the specified ranges of pH and incubated at different temperatures (Kwon et al., 1996). Furthermore, effects of exopolymer dose (0.5 to 39 mg/l) on the flocculation activity were also studied. For HPLC analysis, 1 g of the lyophilized exopolymer was suspended in 10 ml of distilled water. The dissolved bioflocculant was hydrolyzed with 5M HCl at 100°C for 30 min. The solution was neutralized with 2 M Ba(OH)$_2$ and the precipitate was removed. HPLC was performed on the ET300/6.5-Nucleogel sugar Ca-column (300 x 6.5 mm; Macherey-Nagel, Diiren, Germany) with deionized water at a flow rate of 0.5 ml/min at 90°C and 43 bar.

RESULTS AND DISCUSSION

In addition to its biochemical identification described previously by Abd-El-Haleem et al. (2007), transgenic yeast *kloeckera* sp. strain KY1/PHA was subjected for morphological characterization using SEM (Figure 1). It was observed that strain KY1/PHA appeared as an oval with a peripheral budding which confirming its prior identification by ABI20C system. However, the unpredicted exopolymer produced by this strain was characterized, identified and examined as bioflocculant. Both the wild type yeast *Kloeckera* sp. strain KY1 culture broth and its cell-free supernatant were examined as potential bioflocculants. However, no flocculation activity was observed with both samples. As demonstrated in Figure 2, flocculating activity of the culture broth (as a primary
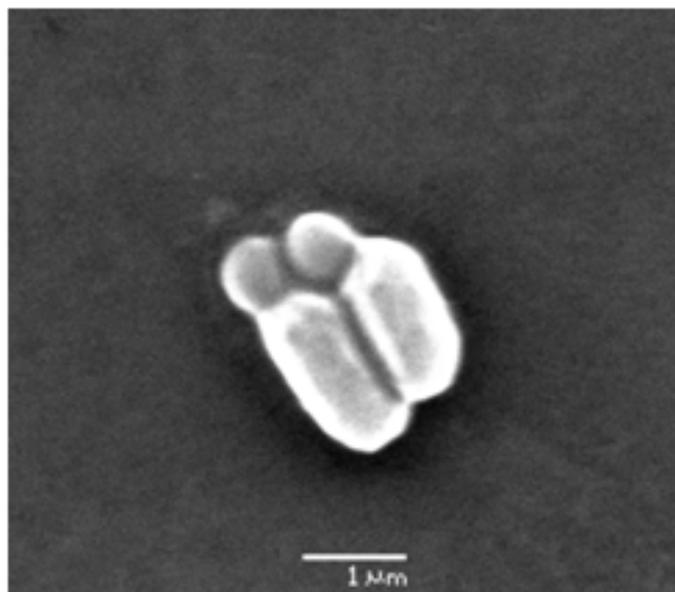
Figure 1. Morphological characterization of strain KY1/PHA using SEM.
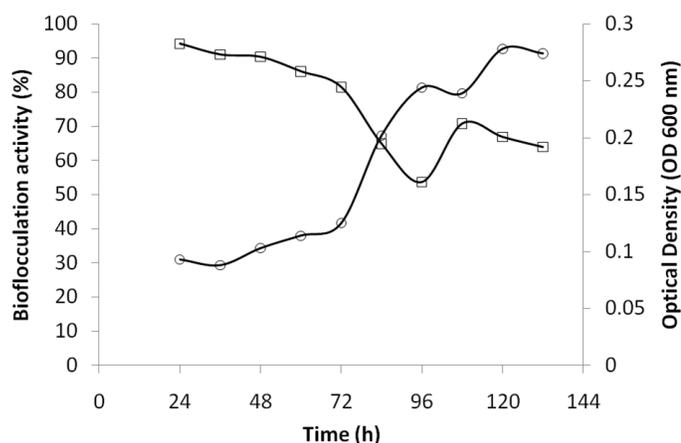

Figure 2. Time course of growth and bioflocculation activity of the transgenic yeast strain KY1/PHA in basal medium.

screening) of transgenic strain KY1/PHA in basal medium reached its maximum (94%) at the first growth phase after 24 h of incubation and then decreased gradually by increasing the growth rate until the end of the experiment (132 h at 30°C), this indicating the degradation of the polymer with time by the transgenic yeast during the growth. However, since the flocculating activity was assayed using the whole culture broth, it was probable that flocculation resulted from both the exopolymer and the cells (Dermlim et al., 1999). It was previously reported that more than 90% of the flocculating activity in the culture broth of *R. erytropolis* was in the supernatant and less than 10% bound to the cells (Kurane et al., 1986). In this s tudy, no significant differences were

observed in the flocculating activity of both culture broth and cell-free supernatant. This may explain that the exo-polymer dissolved in the supernatant was the main factor in bioflocculation process. As shown in Figure 3, the flocculating activity of the exobiopolymer increased proportionally from 1.25 to 13.0 mg/l polymer and reached its maximum at the range from 4.25 (94.2) to 13 mg/l (98.13%). The optimum bioflocculant dosage was 13 mg/l, because the main component of the exopolymer was polysaccharide and protein, the flocculation mechanism might accord with the flocculation hypothesis model by Nakamura et al. (1976).

The best flocculating activity of the exopolymer was recorded at pH 7 (Figure 4). This suggested that the hydroxide ion (OH^-) may interfere with the complex formation of the polysaccharide and kaolin particles mediated by Ca^{2+}, consequently the kaolin particles were suspended in the mixture. The pH range (3 to 9) for flocculation reaction of this polymer was wider than those of the polyglutamate from *Bacillus subtilis* PY-90 (pH range of 3 to 5) and the cationic polysaccharide from *Paecilomyces* sp. I-1 (pH range of 4 to 8) (Takagi and Kadowaki, 1985). Figure 5 deduces that the biopolymer solution is thermally stable over the temperature range of (5 to 50°C). After the biopolymer solution is heated at temperatures above 50ºC, its flocculation efficiency declined. This thermal stability is presumably the main backbone for this biopolymer of polysaccharides (Lu et al., 2005). Higher temperatures may cause degradation of this polysaccharide chain of the biopolymer that reduces its flocculation efficiency. This thermal behavior of the biopolymer is supported by other researchers that studied the thermal stabilities of different bioflocculants (Salehizadeh et al., 2001; Gong et al., 2008). Chemical analysis of the bioflocculant revealed that the proportions of total sugar and total protein content of the biopolymer were 76 and 23% (w/w), respectively, indicating that the bioflocculant was mainly proteoglycan. The HPLC spectrum of the purified bio-flocculant revealed only a single symmetrical peak for fructose which indicated the purity of the bioflocculant. The examined bioflocculant was found to be soluble in water but insoluble in all tested organic solvents. Consequently, it can be recovered from the cell-free supernatant by precipitation with organic solvents such as ethanol (BeMiller and Whistler, 1996; James, 1986)

FTIR spectra (Figure 6) clearly showed the presence of carboxyl, hydroxyl and amide groups in its molecules which are the preferred groups for the flocculation, similar to those observed in polyelectrolytes (Ganesh-Kumar et al., 2004). The presence of the absorption peak at 3436 cm^{-1} suggested the presence of −OH and the C-H stretching band caused the absorption peak at 2949 cm^{-1}. The peak at 1649 cm^{-1} was characteristic of C = O stretching vibration in an amide group. The bands at 1406 cm^{-1} could be assigned to the C = O symmetrical stretching's in the carboxylate, indicating the presence of a carboxyl group in bioflocculant. The peak at 1235 cm^{-1} was an
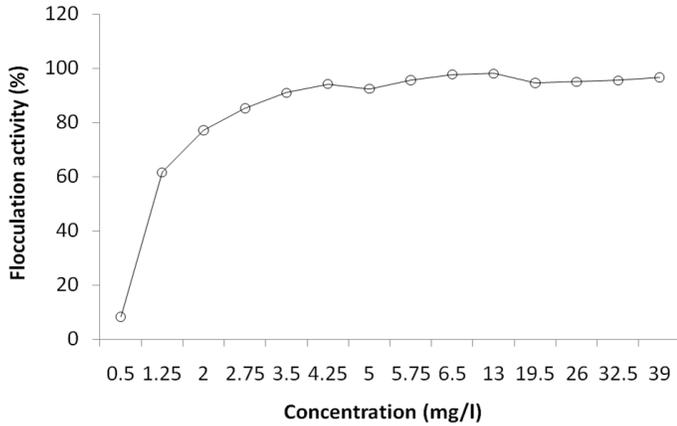
Figure 3. The effect of dosage on the flocculation activity of the partially purified biopolymers from strain KY1/PHA.
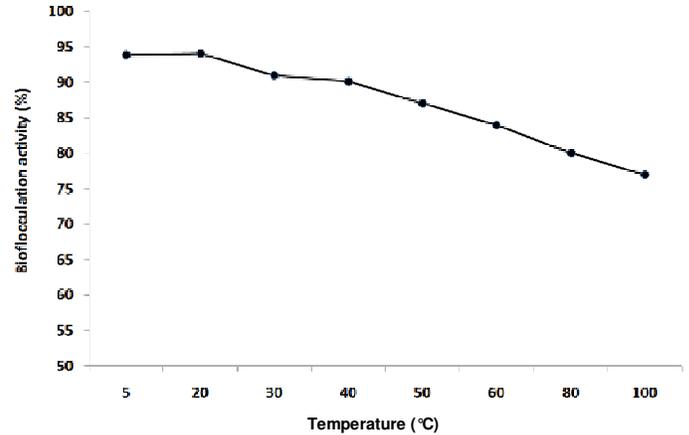
Figure 5. The influence of temperature of Kaolin clay suspensions on the flocculation activity of the partially purified biopolymers from strain KY1/PHA.
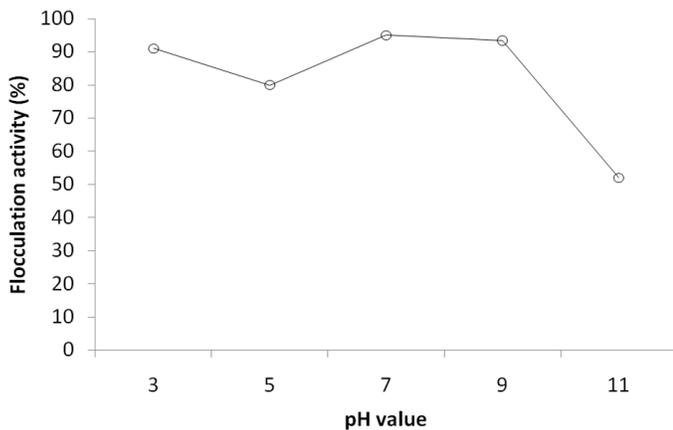
Figure 4. The influence of initial pH of Kaolin clay suspensions on the flocculation activity of the partially purified biopolymers from strain KY1/PHA.
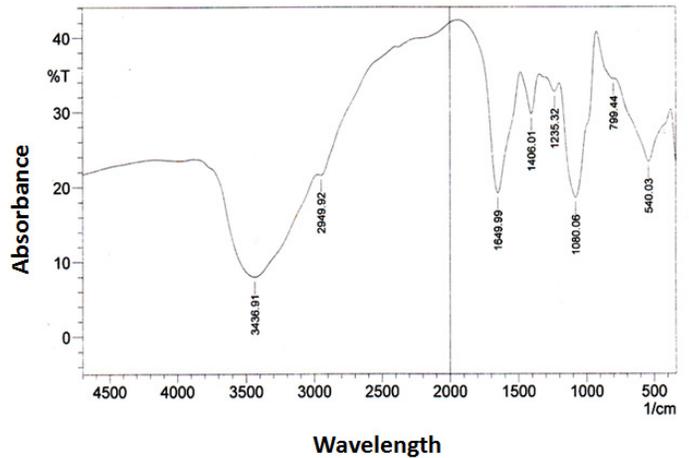
Figure 6. FT-IR spectra of the partially purified biopolymers from of the partially purified biopolymers from strain KY1/PHA.

indication of the S-O symmetrical stretching vibration indicating the presence of a sulfate groups. The absorption peak at 1080 cm^{-1} was known to be characteristic for all sugar derivatives. Interestingly, Figure 7 shows the HPLC analysis of the purified exopolymer and revealed the presence of the monosaccharide fructose which belongs to fructose biopolymers (fructans). Fructans is defined as any compound in which one or more fructosyl-fructose linkages constitute a majority of the linkages (Waterhouse and Catterton, 1993). They are polymers of fructose which are synthesized as storage of carbohydrate around 15% of the flowering plant species (Hendry, 1993). In addition, many microorganisms can produce fructans (Paludan-Muller et al., 2002). There is an increasing research interest in fructans, because of the reported status of fructans, such as inulin, as prebiotics. They are non-digestible food components, which selectively stimulate the growth and/or activity of one or a limited number of bacteria in the colon and thereby

beneficially affect health of the host (Gibson and Roberfroid, 1995).

Conclusions

The unpredicted exopolymer produced by the transgenic yeast strain KY1/PHA was examined as bioflocculant. The advantageous properties of the tested exopolymer such as thermal and pH characteristics, a dosing rate comparable to those of chemical flocculants to flocculate suspended solids and flocculation efficiency, suggest its potential industrial utility. In addition, it was emphasized that due to the complexity of the eukaryotic host system compared to bacteria, introduction of bacterial operon to be expressed in yeast cells may lead to activate synthesis of foreign proteins (Poirier, 2001). Therefore, the second strategy which is undertaken now in our
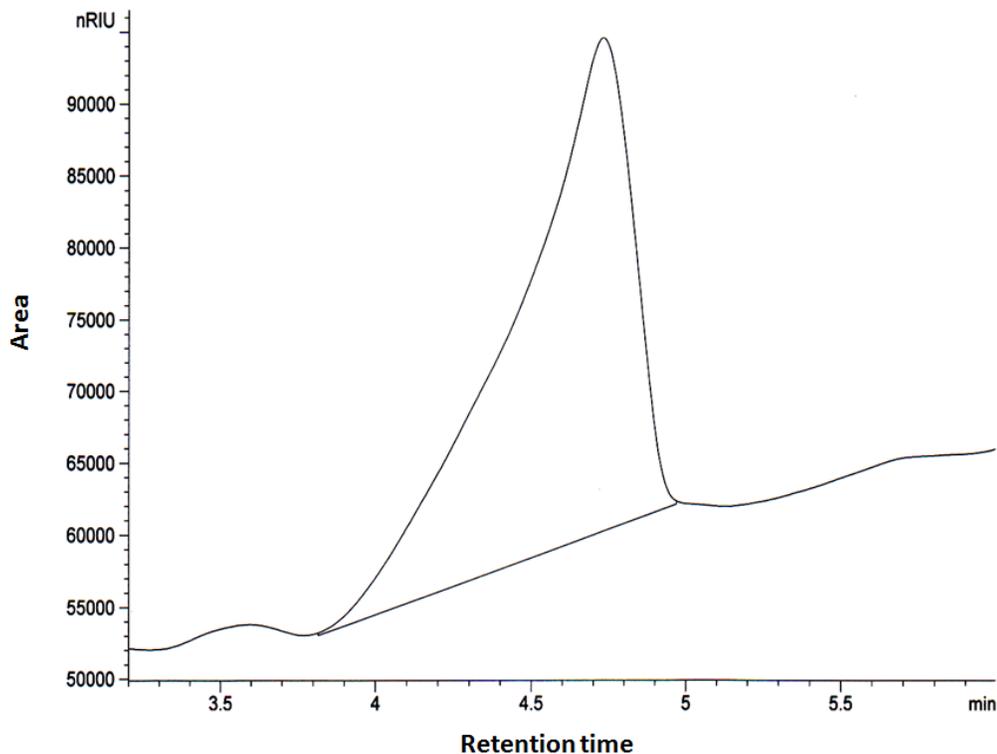
Figure 7. HPLC analysis of the partially purified biopolymers from transgenic strain KY1/PHA**.**

laboratory is focusing on the fundamental explanation of this suddenly occurred phenomenon.

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