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Acidic Exopolysaccharide Flocculant Produced by the Fungus *Mucor rouxii* using Beet-Molasses

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An acidicexopolysaccharide flocculant (EPF), produced by the fungus *Mucor rouxii*, was found to play an important role for protection of cells against abiotic stress such as extreme pH value. This EPF was produced during 48 hrs of growth, at 6% beet-molasses, pH 3.5 and 28^{o}C. The chemical composition of beet-molasses includes heavy metals such as Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} which could be additional stress factors trigger the formation of the EPF. The fungus synthesized this EPF when exposed to low pH, as a form of self-protection. Different carbon sources induces the production of the EPF, however, beet molasses was the most effective and showed maximum production. Ammonium sulfate was the preferred nitrogen source. Higher concentrations of beet molasses depressed fungal growth. The produced EPF exhibited flocculating properties; it possesses good flocculating activity for aggregation and precipitation of charcoal particles where the flocculating activity reached approximately 99%. In addition, it showed maximum flocculating activity at acidic pH values and at high temperature. The main backbone of this EPF is a polysaccharide. Thus, by exploiting beet- molasses as inexpensive carbon source under acidic pH-shock, the fungus produced an EPF characterized by good flocculating properties. It could be utilized in the area of waste-water treatment, in drink water processing as well as in industrial fields.

Keywords: exopolysaccharide, flocculation, *Mucorrouxii*, flocculating activity, beet-molasses.

Introduction:

Microorganisms can synthesize different types of biopolymers such as exopoly-saccharides (EPSs) and bio-flocculants. Polysaccharides are defined by their cellular location, i.e. intracellular (cell wall polysaccharides such as peptidoglycan or teichoic acids) or extracellular-polysaccharides, including the capsule associated with the cell surface and the EPSs secreted in the growth medium. The capsular structure may protect the cell against unfavorable environmental conditions like oxygen tension, toxic compounds, temperature or high osmotic pressure, and may contribute to the uptake of metal ions (Cerning, 1990). Some microorganisms use these biopolymers for attachment to solid surfaces and they sometimes form biofilms, after which these biopolymers can either remain associated with the cell surface or be released into the

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extracellular medium to form amorphous slime (Salehizadeh*et al*. 2000). Some EPSs exhibit flocculating properties. Bio-flocculants are defined as biopolymers that promote flocculating by formation of bridges between them and other particles resulting in the aggregation and precipitation of suspended particles. Thus, bioflocculation is a simple and effective mechanism for the precipitation of suspended solids, colloids and debris by living cells. Generally, when the suspended particles are flocculated into a large floc, they settle down, resulting in a clarified solution and can easily be removed. Bioflocculants can also be used to aid filtration, which is the most crucial step in water purification and in other industrial processes that involve flocculation. The interest in bioflocculant applications is because they are easily biodegradable and harmless to both humans and the environment. Bioflocculants are mostly composed of proteins, polysaccharides, glycoproteins, nucleic acids and some other macromolecular compounds (Salehizadeh *et al*. 2000). Due to their characteristics, these biopolymers have found use in various industries such as in the production of textiles, detergents, adhesives, cosmetics, pharmaceuticals, food additives and brewing. In addition, bioflocculants can also be used in oil recovery, wastewater treatment, dredging and in different downstream process. Some of these biopolymers have also been reported to have anti-tumor, anti-viral and anti-inflammatory activities and can also act as inducers for interferon, and colony stimulating systems (Lin and Zhang, 2004).Recently, due to their extensive capacity for metals, bioflocculants are recommended as surface-active agents for the removal of heavy metals(Lin and Harichund, 2012).

Fungal species belonging to the phylum Zygomycota are an ecologically diverse class of fungi, including both saprophytic and pathogenic fungi of plants, animals, and humans. Some species of the genus Mucor (belonging to Zygomycota) such as *M. racemosus and M.indicus* have been reported as opportunistic human-pathogens which tend to cause Zygomycosis or fungal Pneumonia in patients whose acquired defects in their host defenses (Julie *et al.* 2000). However, other species of Mucor such as *Mucor rouxii* is one of the main species, industrially, used in several traditional fermented foods such as "Tapai" found in East- and Southeast Asia. Moreover, *M. rouxii* is a well-known source for recovery of chitin and chitosan from its cell wall (Tao *et al.*2005), hydrolytic enzymes such as chitin-deacetylase (Araki and Ito 1974), and oleanolic acid derivatives (Capel *etal*. 2011).

Production of polysaccharide bioflocculants from bacteria have extensively reported. However, few research articles are reported by fungi (Nam *et al*. 1996, Krcmar *et al*. 1999, Deng *et al*. 2005, Wang *et al.* 2011). Moreover, limited reports about acidic polysaccharides are mentioned (Kazuaki and Susumu 1990, Kurata *et al*. 2003, Andrade *et al.* 2004, Gang *et al*. 2007). The fungus *M. rouxii* was found to produce an EPF during growth at low pH (3.5) as form of self-protection, with beet-molasses as a carbon source. This feature led us to study the response of the fungus to produce a polysaccharide when exposed to an acidic pH-shock. In the previous work, we studied the production of an EPF by the fungus when exposed to different abiotic stressors (Abdel-Aziz *et al*. 2012) with beet molasses as carbon source. Beet-molasses is extensively used as based-growth medium for many microorganisms (Mao *et al*. 2011). The chemical composition and element content of beet-molasses are represented in Table, 1 (United States).In the present study, optimization of culture conditions for production of an acidic EPF by *M. rouxii* and characterization of its properties were

investigated. To the best of our knowledge, production of an acidic EPF by the fungus *M. rouxii* using beet molasses is reported for the first time.

Table 1. Chemical composition of beet-molasses

Parameter	Concentration
Chemical composition:	
Crude protein	6.30 %
N_2	1.01 %
Organic matter	62.5 %
Moisture	23.5 %
Ash	16.0 %
pH	4.9 - 5.4
Total sugars Sucrose Fructose Glucose	48.30 % 35.9 % 5.6 % 2.6 %
Minerals and Trace elements:	
Calcium	1.3 %
Potassium	4.2 %
Magnesium	0.3 %
Sodium	0.2 %
Copper	14 ppm
Iron	130 ppm
Zinc	8 ppm
Manganese	5 ppm

Materials and Methods

Microorganism and growth conditions

For production of the EPB, *M.rouxii* was grown in beet molasses medium containing (g/L): 60 beet-molasses as a carbon source and, 1.5 $(NH_4)_2SO_4$ as a nitrogen source. All cultures were performed in conical flasks 250-ml containing 50 ml culture medium under shaking conditions (130 rpm) at pH 3.5 and 28^{o}C for 5 days. Flasks were inoculated with a pre-culture (7 days) of the fungus *M. rouxii*, grown on potato dextrose agar. Extraction and purification of the EPF were performed as reported by (Wang *et al*. 2011).

Effect of carbon and nitrogen source

Effects of carbon and nitrogen sources on production of the EPF were assessed. Different carbon sources including glucose, sucrose, fructose, maltose, galactose as well as beet molasses and sugarcane bagasse were tested for production of the EPB. Organic nitrogen sources (*viz*., peptone, tryptone, urea, yeast extract and casein), and inorganic nitrogen sources (*viz*., ammonium chloride, ammonium sulphate and ammonium carbonate) were also tested for their effect on the EPB production.

Effect of initial pH

The effect of initial pH of medium on production of the EPF was investigated. Different carbon sources were tested along with different pH values to assess the effect of pH. The initial pH of the culture medium varied in a range from 2.5 – 6.5 using 0.1 M HCl or NaOH.

Effect of molasses concentrations

Production of the EPB was tested using different concentrations of beet molasses; 2, 4, 6, and 8 %. The produced EPB was expressed by detection of the flocculating activity.

Thermal-stability

The effect of heat on the crude and purified EPF was assessed according to the method of Gong *et al*. (2008). Both of EPF were placed in a water bath and heated over a period of 45 min, after which, 2 mL of the EPF was withdrawn and assessed for residual flocculating activity.

Detection of the flocculating activity

The produced EPF was expressed by detection of the flocculating activity (FA) using charcoal as the test suspension as described by Zhang *et al.* (2002). Briefly, 0.2 mL of cell free supernatant was added to 5 ml of charcoal suspended solution (5 g/L)in a test tube. The mixture was allowed to be stand for 5 min. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. A control

experiment was prepared using the same method, but using fresh culture medium (B). The flocculating activity was calculated according to the equation:

Flocculating activity (%) = [(B − A)/A] × 100where, A is the optical density of the sample at 550 nm; B is the optical density of control experiment at 550 nm.

Chemical analysis

Protein content of the EPF was detected by the method of Lowry (Lowry *et al*. 1951). The content of amino-sugars in the EPF was measured by the Elson-Morgan method (Chaplin and Kennedy, 1986), using glucosamine as the standard. Sugars content was determined by the method of Dubois *et al* (1956).The average molecular weight of the EPF in relation to the viscosity was calculated according to the method of Il'ina *et al.* (2001). The viscous EPF were analyzed for determination of the functional groups by the infrared using a FT-IR- Raman (Nexus 670, Nicolet-Madison-WI-USA). The spectrum of the sample was recorded on the spectrophotometer over a wave number range 4000-400 cm^{-1}. The IR-spectra of the produced EPF was detected by the "Central Services Laboratory", National Research Center.

Results

Growth of the fungus under normal conditions

As shown in Table 1, beet-molasses contain mainly glucose, sucrose, fructose as well as minerals and portions of heavy metals (Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}). *M. rouxii* grew well in molasses-based medium without any pretreatment for molasses; low production of the EPF and low viscosity in culture broth were observed under normal conditions (Fig. 1a). However, at a concentration of 6% beet-molasses (pH 3.5, and at 28°C), high production of the EPF accompanied with increased viscosity was occurred throughout the lag to the stationary phases (Fig. 1b). Worthy mention is that, cells of *M. rouxii* form pellets during normal growth, except under stress conditions; the cells extremely aggregated and form flocs during 48 hrs as a form of self-protection for fungal cells, with obvious drop in cell growth (Fig. 1b).

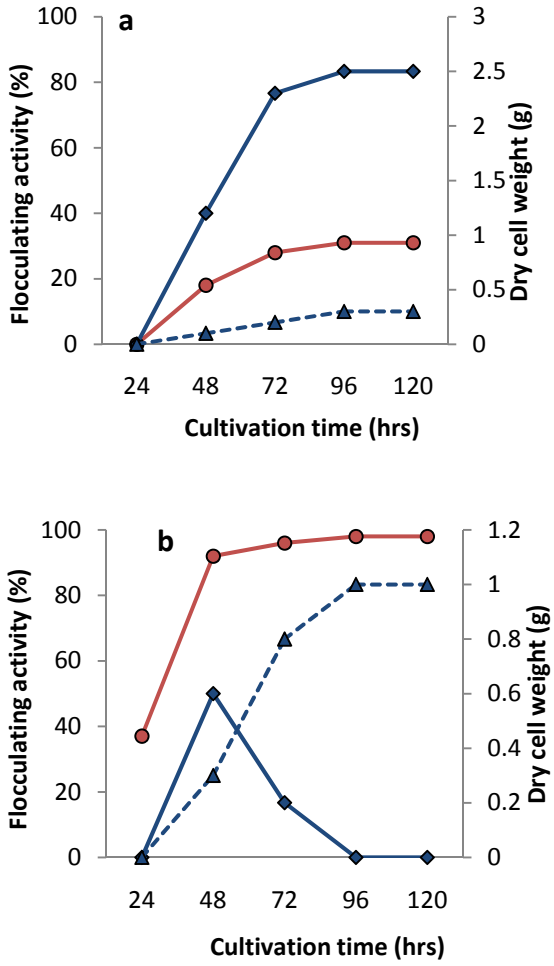

Fig. 1. Growth(♦) of *Mucor rouxii*; in beet-molasses medium, the flocculating activity % (●), and the viscosity (▲) during normal growth; 2% molasses (a) and growth under stress of high beet molasses concentration; 6 % and pH 3.5(b).

Effect of carbon and nitrogen sources

Figure 2, shows the effect of different carbon (60 g/L) sources on EPF production. The fungus utilized all carbon sources at pH 3.5 but higher production of the EPF was

observed with beet molasses at pH 3.5-4.5, accompanied with a flocculating activity exceeded 98%. Beet molasses was used for all subsequent cultures. The fungus poorly utilized various organic and inorganic nitrogen sources (1.5 g/L) resulting in production of EPF with flocculating activities ranging from about 12% to 29% (Fig. 3), except for yeast extract (organic) and ammonium sulfate (inorganic) nitrogen sources which resulted in FA of about 80 and 95%.

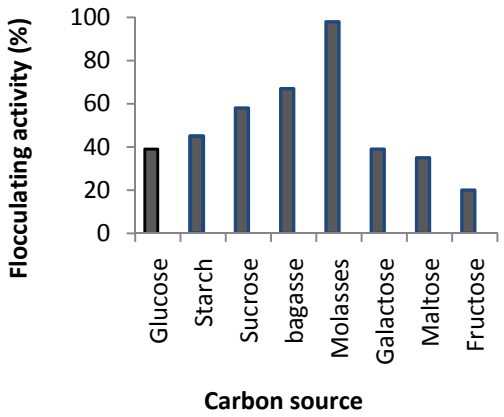

Fig. 2. Effect of different carbon sources on production of the exopolysaccharide flocculant by *Mucor rouxii*. Carbon sources were added at a concentration of 6% at pH 3.5.

Effect of initial pH

Effect of the initial pH of medium on the EPF production was investigated. The fungus poorly utilized all carbon sources at different pH values except for pH 3.5 and 4.5 (Fig. 4),where the highest FA reached, approxi-mately, 99 and 95%at acidic pH of 3.5 and 4.5, respectively with beet molasses as carbon source. Weakly alkaline pH (6.5) was unfavorable (Fig. 4) for the EPF production by the fungus.

Effect of molasses concentrations

Maximum production of the EPF was obtained at concentrations of 4-6 % beet molasses (Fig. 5), whereas, at 8 % beet molasses in the culture medium, production of the EPF was obviously depressed.

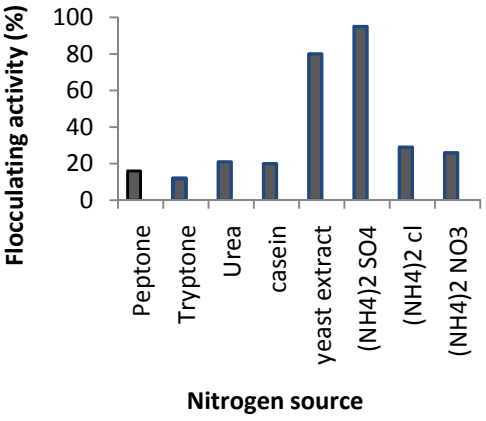

Fig. 3. Effect of different nitrogen sources on production of the exopolysaccharide flocculant by *Mucor rouxii*. Nitrogen sources were added at a concentration of 0.15 %, with beet molasses (6%) and at pH 3.5.

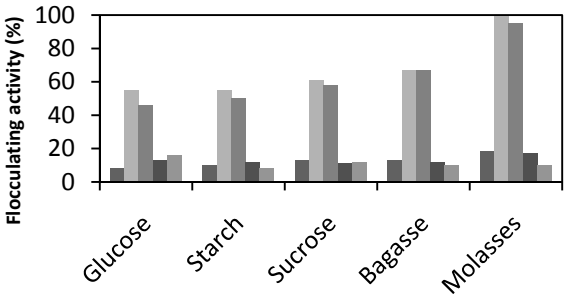

Fig. 4. Effect of initial pH on production of the exopolysaccharideflocculant by *Mucor rouxii*. Carbon sources were used at a concentration of 6% at different pH values: (■)2.5, (■)3.5, (■) 4.5, (■)5.5, and (■) 6.5.

Distribution of the exopolysaccharide flocculant

Under optimized culture conditions (6% beet-molasses, 0.15 % $(NH_4)_2SO_4$, pH 3.5 at 28^{o}C for 72hrs), 200 ml of a clear culture broth was obtained. The FA of the clear culture reached 98.7%, thus it could directly be used in practice for further studies. To investigate the distribution of FA of the EPF

during the recovery procedures, each of the FA of the culture broth, the cell–free supernatant, and the supernatant after ethanol precipitation was detected. The FA of culture broth was almost the same as that of its cell-free supernatant, whereas the supernatant after ethanol precipitation showed no flocculating activity (data not shown). These results indicate that the EPF is produced extracellularly by the fungus.

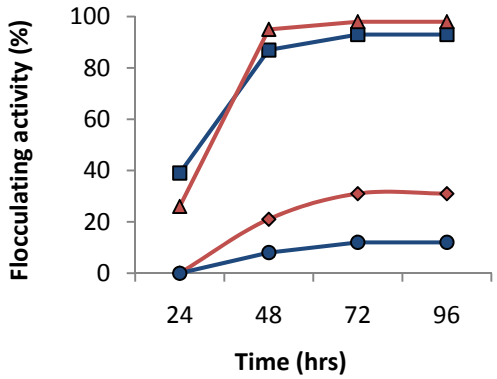

Fig.5: Effect of molasses concentrations on production of the exopolysaccharide flocculant by *Mucor rouxii*. Molasses were added at concentrations of: (♦)2%, (■) 4%, (▲) 6 %, and (●)8 % at pH 3.5.

Some properties of the exopolysaccharide flocculant

The flocculating activity property

The crude EPF produced by *M. rouxii* was found to possess good FA property. Particles of soil and charcoal were effectively aggregated and precipitated by addition of the crude EPF through 5 min(Fig. 6). With charcoal as the suspended particles, the FA of the crude supernatant reached, approximately, 99% without addition of cations such as Ca^{2+} and Mg^{2+}. However, addition of Ca^{2+} or Mg^{2+} was found to be necessary to achieve maximum FA when the culture supernatant was tested to precipitate soil particles (Abdel-Aziz *et al*. 2012). Addition of trivalent cations such as Al^{3+} and Fe^{3+} completely inhibited activity of the EPF as indicated by reduction in FA (Fig. 7).Divalent cations such as Mn^{2+}, Cu^{2+}, or Co^{2+}slightly affected the FA, whereas PB^{2+} inhibited the FA (Fig. 7).On other hand, it was found that higher or lower dosage of the EPF resulted in low flocculating efficiency.

Fig. 6. Efficacy of the crude exopoly-saccharide flocculant produced by *Mucor rouxii* in aggregation and precipitation of particles: soil (left image) and charcoal (right image). An image showed the precipitating action (right) in comparison with the control (lift).

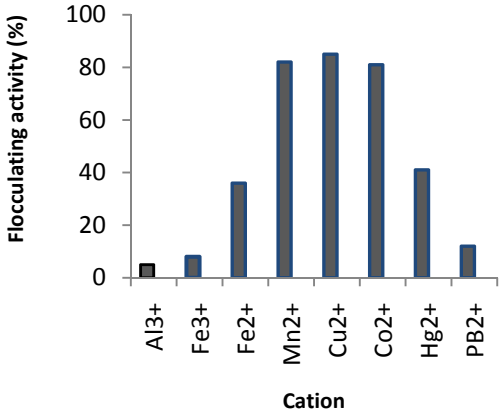

Fig. 7.Effect of cations on the flocculating activity of the crude exopolysaccharide flocculant. The indicated cations were used as chlorides; 0.25 ml of 8 mM cation was added to a reaction mixture containing charcoal particles.

Effect of pH and temperature

The effect of different pH values of reaction mixture on the FA was examined. A reaction mixture, containing charcoal suspension and

10 or 2 ml/L of the crude or purified EPF, were adjusted with HCl or NaOH and then the FA was measured. As shown in Fig. 8(a), high FA was detected in an acidic pH range of 3.0-4.0, indicating that the EPF produced by *M. rouxii* is a novel acidic polysaccharide. Effect of temperature on the FA revealed that, maximum activity (Fig. 8,b) was occurred at 60-80°C for both the crude and purified EPF.

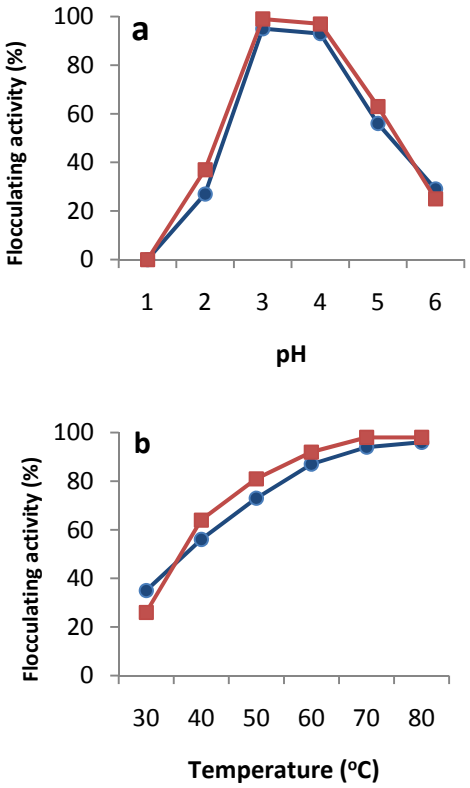

Fig. 8. Effects of pH (a) and temperature (b) on flocculating activity of the crude (•) and purified (■) exopolysaccharideflocculant.

Thermal stability

Heating up to 100°C for 45 min does not significantly affect the flocculating activity of the EPF; the purified EPF remained most of its activity suggesting it to be thermo-stable (data not shown). It is interested to study the stability of the purified EPF against degradation by enzymes. A variety of purified enzymes (chitinase, chitosanase, and *B*, 1-4 glucanase) produced by *Bacillusalvei*NRC-14 were tested for degradation of the EPF, individually or in combination (Abdel-Aziz *et al.* 2012). Results revealed that: firstly, individual use of chitosanase or *B*,1-4 glucanase resulted in an obvious degradation of the EPF. Secondly, an efficient degradation of the EPF was achieved when a mixture of these enzymes was used (Abdel-Aziz *et al.* 2012). The crude EPF was also found to be stable against degradation by enzymes. Worthy mention is that, the EPF produced by *M. rouxii* contains mainly polysaccharides as well as urinate and minor proteins. The molecular weight of the EPF was calculated to be 1.78 x 10^6 Da.

IR-spectra

The IR-spectra was performed by the purified exopolysaccharide. The IR-spectra showed different absorption bands and peaks, characteristic of carbohydrate, carboxyl, urinate and hydroxyl groups (Fig. 9).

Discussion

Abiotic stress factors are known to enhance secondary metabolites production and there have been a number of studies on the application of an environmental stimulus for the enhancement of productivity. These environmental stimuli include heat shock, cold shock, acidic or alkali shock (Vijayabaskar *et al*. 2011). In their natural environment, microorganisms adapt to stressful situations. A characteristic of this adaptation is the induction of stress proteins which serve as protective function allowing adaptation to stresses, regardless of the nature of the stress factor (Kim *et al*. 2000). During an experiment for production of chitosan from cell wall of the fungus *M. rouxii*, a culture broth was found to be highly viscous and displayed flocculating properties (at pH 3.5, using untreated beet-molasses). The fungal cells were extremely aggregated and surrounded by a biofilm. It

was concluded that an acidic pH-shock may induce the formation of an EPF by the fungus as a form of self-protection (Abdel-Aziz *et al*. 2012). In the present study, we optimized the culture conditions for production of EPF by the fungus when exposed to abiotic stress (low pH). Presence of Mn^{2+}, Fe^{2+}, and Zn^{2+}in beet-molasses could be additional stressors resulted in triggering the production of the EPF to protect fungal cells. Quick formation of the EPF by *M. rouxii* after 48 hrs of growth at pH 3.5 may confirm this assumption.

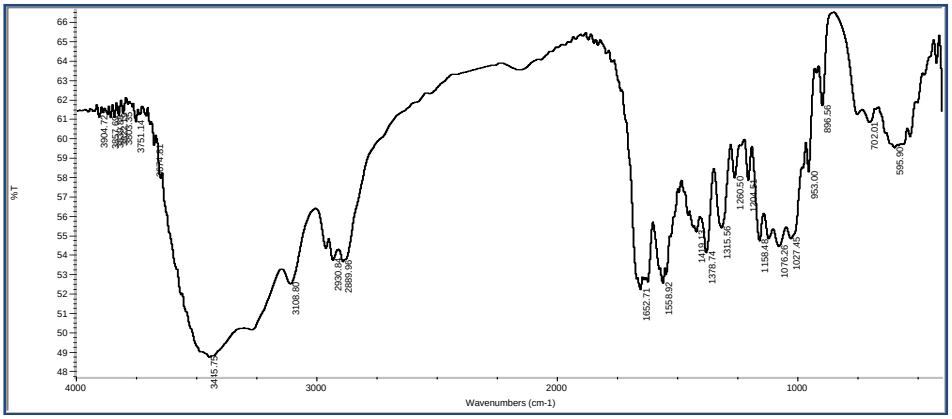

Fig. 9. IR-spectra of the exopolysacchride flocculant produced by *Mucor rouxii.*

Regarding the effect of carbon sources on EPF production, the fungus utilized all carbon sources at pH 3.5 and 4.5 but maximum production (expressed as flocculating activity) of 98.7% was occurred with beet molasses. The fungus poorly utilized all carbon sources at other pH values. These results indicate that, chemical composition of beet molasses especially presence of heavy metals and the lower pH of the growth medium may induce the formation of an EPF by the fungus to protect fungal cells. With respect to the nitrogen source, it is an important nutrient factor for enhancing EPSs production. Most microorganisms utilize either organic or inorganic nitrogen sources, or both, to produce EPS bioflocculant. In this study, yeast extract or ammonium sulfate [$(NH_4)_2SO_4$] favored the production of the EPF, however, ammonium sulfate was the most preferred, resulting in flocculating activity of more than 95%. Other organic or inorganic nitrogen sources were poorly utilized.

Effect of pH on the EPF production differed widely according to the microorganism. In the present study, effect of pH of culture medium revealed that, low pH values trigger the EPF production. The lower pH along with the chemical composition of molasses induces production of the EPF under abiotic stress conditions, and aggregation of fungal cells as well as formation of biopolymer flocculant could be a reaction against the abiotic stress conditions. Shimofuruya *et al.* (1995) reported that, *Streptomycete griseus* produced a bioflocculant under acidic pH conditions. The mycelial growth of *Aspergillus sojae* is enhanced when the pH of the culture is controlled at 6 and no flocculating activity is observed in cultures grown at pH 8 (Nakamura *et al.* 1976). The flocculant production by *Rhodococcus erythropolis*is is higher at alkaline pH values of 8.0 - 9.5 than at other pH values (Kurane and Tomizuka 1992) Thus, biopolymer production is differed widely (Kim *et al.* 2008) according to the microorganism.

The concentration of beet molasses greatly affected the fungal growth and formation of the EPF. During normal growth (1-2%, beet molasses), fungal growth increased steadily, until it peaked after 72 hrs (FA of 31.4%) with less viscosity (Fig.1a). With higher concentrations of beet molasses (6%), the FA increased to 98.7% after 48 hrs, at which fungal growth declined (Fig. 1b) and forming a block of fungal cells surrounded by a biofilm after exposure to an acidic stress as a form of self-protection. This trend suggests that the EPF was produced by biosynthesis but not after autolysis, and the decline in growth after 48 hrs along with increasing the FA could not be due to cell autolysis comparing with normal growth which continued up to 120 hrs (Fig.1a). In addition, the FA did not decrease up to 120 hrs indicating the stability of the EPF (Abdel-Aziz *et al.* 2012) against any bioflocculant-degrading enzymes. Some properties of the EPF were investigated. Both the crude and purified EPF showed excellent flocculating properties for aggregation and precipitation of soil and charcoal particles. Moreover, heavy metals such as Mn^{2+}, Cu^{2+} and Co^{2+} slightly affected the FA. On other hand, Al^{3+}, Fe^{3+} and PB^{2+} completely inhibited activity of the EPF. Metal ions either stimulate or inhibit bioflocculant production. Among the mechanisms proposed for stimulation are: 1) neutralization and stabilization of the residual charge of functional group on the bioflocculant by the metal ions (Kwon *et al*. 1996); and 2) increase in ionic strength of the suspension solution as a result of addition of metal ion; thereby, decreasing electrostatic forces of the suspended particles (Wang *et al.* 2011).Some exopolysaccharide flocculants need Fe^{2+} and Zn^{2+} to show higher FA (Lu *et al.* 2005), whereas, the presence of $Al^{3+}$and Fe^{3+} decreased the FA of the EPS flocculant from *Bacillus* sp. AS-101 (Salehizadeh*et al.* 2000). Divalent cations (Ca^{2+}, Fe^{2+}) were found to be more effective in stimulating flocculating activity than monovalent (K^{+}) and trivalent (Fe^{3+}) cations (Ntsaluba*et al*. 2011).Generally, EPS bioflocculants have been successfully tested in waste-water treatment (Gong *et al.* 2008), clarification (Todd *et al*. 2010), dye removing (Mao *et al.* 2011), and heavy metal removing (Lin and Harichund 2012).

Both the crude and purified EPF exhibited maximum FA at acidic pH values and in temperature ranging from 60-80^{o}C and exceeded. The produced EPF was found to be thermo-stable. This thermal stability might be due to its structure as a polysaccharide (Lu *et al*. 2005). The content of uronic acid in a polysaccharide bioflocculant will provide large quantities of carboxyl and hydroxyl functional groups in the polysaccharide backbone, which will interact with each other to create a considerable amount of hydrogen bonds within the molecule that necessary for thermal stability of a bioflocculant (Anthony *et al.* 2012).

The MW of the EPF produced by *M. rouxii* was found to be 1.78 x 10^{6} Da (Abdel-Aziz *et al*. 2012). The MW and functional groups in the molecular chains are important factors for the flocculating activity by a bioflocculant. For protein bioflocculants, the amino and carboxyl groups are the effective groups for flocculation, but their molecular weights are usually low. In contrast, polysaccharide bioflocculants have high molecular weights and many functional groups caused the aggregation of microbial cells and other impurities in solutions. The EPS bioflocculant from *M. rouxii* has good flocculating capability; many particles could adsorb to a long molecular chain, and the particles adsorbed on the chain could be adsorbed simultaneously by other flocculant chains. These properties lead to the formation of three-dimensional flocs that are capable of settling fast (Zhang *et al*. 2002). Higher or lower dosage of the EPF flocculant produced by *M. rouxii* resulted in low

flocculating efficiency. When the dosage is insufficient, the bridging phenomena can't be effectively formed. Whereas, over addition of dosage cause competition and repulsion of negatively charged particles, leading to re-suspension of particles (Gong *et al.* 2008).

The spectrum of the purified EPF was determined (Fig. 9). The IR-spectra showed absorption bands and peaks characteristic of carbohydrate, carboxylate, and hydroxyl groups as well as urinate and C-O-C ester linkages. The spectrum of the purified EPF showed an absorption band at 3445 cm^{-1}, which is characteristic of a hydroxyl group. A minor band at 2930 cm^{-1}, known to be typical of carbohydrates, indicated C-H asymmetrical stretching vibration. An asymmetrical stretching peak observed at 1652 cm^{-1} is characteristic of C=O stretching vibration in $-NHCOCH_3$group. The band observed at 1419 cm^{-1}, indicated the presence of urinate. The spectrum also displayed a minor peak at 1378 cm^{-1}, indicating the presence of carboxylate in the polymer. In addition, the absorption band at 1076 cm^{-1} indicated asymmetrical stretching vibration of a C-O-C ester linkage. The small absorption band at 896 cm^{-1} could be associated with *B*-glycosidic linkages between the sugar monomers. The absorption peaks around 1000-1100 cm^{-1} are known to be characteristic for all sugar derivatives. The IR-spectrum, in our study, is in consistent with the results reported previously (Xiong*et al*. 2010, Lin and Haeichund 2011). Thus, the EPF from *M. rouxii* contains important functional groups such as carboxyl and hydroxyl. The carboxyl groups present on the molecular chain make the chain stretched-out because of electrostatic repulsion, and the stretched molecular chains provide more effective sites for particle attachment (Zhang *et al.* 2002). Furthermore, carboxylate groups act as non-specific ion-exchange material which may convey chelating property (Lin and Haeichund 2011).In addition, presence of hydroxyl group within the polymer favored the possibility of hydrogen bonding with one or more water molecules (Kwon *et al.* 1996). Presence of urinate assists metal uptake and allows for adhesion of microorganisms to surfaces. A major condition for flocculation is that the molecules of bioflocculants could adsorb onto the surface of particles; when bioflocculants are approaching in solutions, an attractive force must exceed the electrostatic repulsion force (Zhang *et al*. 2002).

Depending on specific environmental conditions, microorganisms can produce EPSs of particular composition and physiochemical properties and this promotes the survival of microbial populations. Stress is a change in the genome, leading to a decrease in the growth rate or survival. Stress responses are of particular importance for microorganisms, because their habitats are subject to continual changes. Some microorganisms have the ability to synthesize EPSs which are often thought to have a protective function for microbial cells against abiotic stress (Vorob'eva 2004). Bacteria and fungi are remarkable in that they are able to survive, grow, respond, and adapt to adverse environmental stressors such as heat or cold, high or low pH, antimicrobials or even the human immune system, by changes in gene expression, ensuring their survival. When the stress exceeds, bacteria and fungi sense and convert extracellular physical or chemical environmental stimuli into a specific cellular response, resulting in altered gene expression and enzyme activities to pass on, survive, and resist abiotic stress (Jörg 2011). To date, various biopolymers have been produced from different microorganisms. These biopolymers are, generally, high molecular weight polymers

and are presumed to be complex hetero-polymers, proteins, glycoproteins, glycolipids (Salehizadeh and Shojaosadati, 2001), or polysaccharides (Lin and Haeichund, 2011). Production of such biopolymers requires expensive substrate(s), leading to the problem of high production costs. In the present study, inexpensive substrate was used, exploiting beet-molasses as a by-product of sugarcane industry to reduce the production costs.

Conclusion:

This study has shown the fungus *M.rouxii* to be a good source of a new acidic thermostable exopolysaccharide flocculant. This is the first report implicating the fungus *M. rouxii* in EPF production.

Based on data obtained, the following conclusions could be drawn:

1) Beet-molasses, a by-product generated from sugarcane industry, could be exploited for production of an EPF by the fungus *M. rouxii*.

2) It is effectively used as low-cost substrate without any pre-treatment.

3) The exopolysaccharide flocculant was found to be synthesized by the fungus due to exposure to abiotic stress of an acidic pH-shock induced the biosynthesis of this EPF as a form of self-protection by the fungus. Higher concentrations of beet molasses (6%) are considered to be an additional stress factor.

4) In our studies, the produced EPF was found to possess excellent flocculating activity, highly stable against biodegradation by enzymes, and need low- cost nutritional medium.

References

Abdel-Aziz SM, Hamed H, Mouafi F, and Gad A. 2012. Acidic pH-shock induces the production of an exopolysaccharide by the fungus *Mucor rouxii:* utilization of beet molasses. New York Sci.J., 5: 52-61.

Andrade L, Salgado L, Farina M, *et al*.2004. Ultrastructure of acidic polysaccharides from the cell walls of brown algae. J. Struct. Biol., 145: 216-225.

Anthony U, Cosa s, Leonard M, *et al.* 2012. Thermostable bacterial bioflocculant produced by *Cobetia* spp. isolated from Algoa Bay (South Africa). Int. J. Env. Res. Pub. Health, 9: 2108-2120.

Araki Y. and Ito E. 1974. A pathway of chitosan formation in *Mucorrouxii*: enzymatic deacetylation of chitin. Biochem. Biophys. Res. Commun., 56: 669-675.

Capel C, Souza A, Carvalho T. *et al*. 2011.Biotransformation using *Mucor rouxii* for the production of oleanolic acid derivatives and their antimicrobial activity against oral pathogens. J. Ind. Microbiol. Biotechnol., 38: 1493-1498.

Cerning J**.** 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev., 87: 113-130.

Chaplin M, and Kennedy J. 1986. Carbohydrate analysis. IRL Press, Washington, DC, pp. 2-5.

Deng S, Yu G, and Ting Y. 2005. Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. Colloids and Surfaces B: Biointerfaces, 44: 179-168.

Dubois M, Gilles K, Hamilton J, Rebers P, and Smith, F. 1956.Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350–356.

Gang X, Igor A, and Mark T. 2007. Immunomodulatory activity of acidic polysaccharides isolated from *Tanacetum vulgare.* Int. Immuno-pharmacol., 7: 1639–1650.

Gong W, Wang S, Sun X, Liu X, Yue Q, and Gao B. 2008.Bioflocculant Production by culture of *Serratia ficaria* and its application in wastewater treatment. Biores. Technol., 99: 4668-4674.

Il'ina A, Varlamov V, Melent A, and Aktoganov G. 2001. Depolymerization of

chitosan with the chitinolytic complex from bacteria of the genus *Bacillus* sp.739. Appl. Biochem. Microbiol., 37: 160-163.

Jörg O. 2011. *Environmental stimuli*. KIT-University of the State of Baden-Wuerttemberg and National Research Center of the Helmholtz Association.

Julie A, Carolyn L, Vanover S, and Doris J. 2000. Zygomycetes in Human Disease. Clin. Microbiol. Rev., 13: 236-301.

Kazuaki N, and Susumu O. 1990.Effects of nitrogen source on the production of acidic polysaccharide by a benzalkonium chloride. Agric. Biol Chem., 54: 2725-2726.

Kim C, Chang Y, and Chun G. 2000.Enhancement of kasugamycin production by the pH shock in batch cultures of *Streptomyces kasugoensis*. Biotechnol. Prog., 16: 548-552.

Kim Y, Moon M, Song J, *et al*. 2008.Acidic pH shock induces the expression of a wide range of stress-response genes. BMC Genomics, 9: 604-613.

Krcmar P, Novotny C, Marais M, and Joseleau J. 1999.Structure of extracellular polysaccharide produced by lignin-degrading fungus *Phlebia radiata* in liquid culture. Intr. J. Biolog. Macromol., 24: 61-64.

Kurane R, Hatumochi, K, Kakuno T, *et al*. 1994. Purification and characterization of lipid bioflocculant produced by *Rhodococcus erythropolis*. Biosci. Biotechnol. Biochem., 58: 1977-1982.

Kurane R, and Tomizuka N. 1992. Towards new biomaterial produced by micro-organism-bioflocculant and bioabsorbent. Nippo Kagaku Kaishi, 5, 453-463.

Kurata S, Yamada K, Takatsu K, *et al*. 2003.Extracellular acidic polysaccharide production by a two-membered bacterial coculture. Biosci. Biotechnol. Biochem., 67: 8-14.

Kwon G, Moon S, Hong S, Lee H, Kim H, and Yoon B. 1996. A novel flocculant biopolymer produced by *Pestalotiopsis* sp. KCTC-8637P. Biotechnol. Lett., 18: 1459-1464.

Lin J, and Harichund C. 2011. Isolation and characterization of heavy metal removing bacterial bioflocculants. Afr. J. Microbiol. Res., 5, 599-607.

Lin J, and Harichund C. 2012. Production and characterization of heavy-metal removing bacterial bioflocculants. Afr. J. biotechnol., 11: 9619-9629.

Lin Z, and Zhang H. 2004. Anti-tumor and immune-regulatory activities of *Ganodermalucidum* and its possible mechanisms. Acta. Pharmacol. Sin., 25: 1387-1395.

Lowry O, Rosebrough N, Farr A, and Randall R. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Lu W, Zhang T, Zhang D, *et al*. 2005. A novel bioflocculant produced by *Entero-bacter aerogenes* and its use in defecating the trona suspension. Biochem. Eng., *J*. 27: 1–7.

Mao Y, Tian C, Zhu J, Zhang T, and Tong L. 2011. Production of a novel bioflocculant by culture of *Bacillus cereus* B-11 using molasses wastewater and its use for dye removal. Adv. Mater. Res., 230: 1119-1122.

Nakamura J, Miyashiro S, Hirose Y. 1976. Conditions of production of microbial cell flocculant by *Aspergillus sojae* AJ-7002. Agric. Biol. Chem., 40: 1341-1347.

Nam J, Kwon G, Lee S, *et al*. 1996. Bioflocculant produced by *Aspergillus sp*. JS-42. Biosci. Biotechnol. Biochem., 60: 235-237.

NtsalubaL, Oladele A, Leonard M, and Anthony O. 2011. Studies on bioflocculant production by Methylo-bacterium sp. Obi isolated from a freshwater environment in South Africa. Afr. J. Microbiol. Res., 5: 4533-4540.

Salehizadeh H, Vossoughi M, and Alemzadeh I. 2000. Some investigations

on bioflocculant producing bacteria. Biochem. Eng., 5: 39-44.

SalehizadehH, and Shojaosadati S. 2001. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. Biotechnol. Adv., 19: 371-385.

Shimofuruya H, Koide A, Shirota K, *et al*. 1995. The production of flocculating substance(s) by *Streptomyces griseus*. Biosci. Biotechnol. Biochem., 60: 498–500.

Tao W, Svetlana Z, Ann F, *et al.* 2005. Physiochemical properties and bioactivity of fungal chitin and chitosan. J. Agric. Food Chem., 53: 3888-3894.

Todd M, Anderson J, Lane S, and Waddell E. 2010.Polyelectrolyte flocculation of grain stillage for improved clarification and water recovery within bioethanol production facilities. Biores. Technol., 101: 2280-2286.

Vijayabaskar P, Babinastarlin S, Shankar T. *et al*. 2011. Quantification and characterization of exopolysaccharides from *Bacillus subtilis* (MTCC 121). Adv. Biolog. Res., 62: 71-76.

Vorob'eva L. 2004. Stressors, stress reactions, and survival of bacteria: A Review. Prikl. Biokhim., 40: 261-269.

WangL, Ma F, Qu Y, *et al*. 2011. Characterization of a compound bioflocculant produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6. World J. Microbiol. Biotechnol., 27: 2559–2565.

Xiong Y, Wang Y, Yi Y. *et al.* 2010. Production and Characterization of a novel bioflocculant from *Bacillus licheniformis.* Appl. Env. Microbiol., 76: 2778-2782.

Zhang J, Wang R, Jiang P, and Lui Z. 2002. Production of an exopolysaccharide bioflocculant by *Sorangium cellulosum*. Lett. Appl. Microbiol., 34: 178-181.