Acidic pH-Shock Induces the Production of an Exopolysaccharide by the Fungus *Mucor rouxii*: Utilization of Beet-Molasses

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Abstract: Depending on specific environmental conditions, microorganisms can produce exopolysaccharides (EPSs) of particular composition and physiochemical properties, and this promotes the survival of microbial populations. An extracellular exopolysaccharide (EPS), synthesized by the fungus *Mucor rouxii,* was found to play an important role for the protection of cells against abiotic stress such as extreme pH values or elevated temperature. This EPS was produced during 48 hr of growth, at pH 3.5 and 28°C using beet-molasses as a low-cost substrate. The chemical composition of beet-molasses includes high concentrations of K^+, Na^+, Fe^{2+}, and Zn^{2+} which could be additional stress factors trigger the formation of the EPS. The molecular weight of the EPS was found to be 1.78 x 10^6 Da, and it had good flocculating activity for precipitation and aggregation of soil and charcoal particles. The main backbone of this EPS is a polysaccharide. The infrared spectra analysis showed the presence of urinate, hydroxyl, and carboxyl groups which are the important factors for the flocculating activity of a bioflocculant. This work is focused on studying the response of the fungus *M. rouxii* to produce an EPS under abiotic stress condition. An acidic pH-shock was found to be the strongest stressor for synthesizing the EPS, which showed flocculating activity of approximately 99%, exploiting beet- molasses as inexpensive carbon source. The produced EPS showed good flocculating activity, higher stability against enzymatic degradation, capability for metal removing, and is heat-stable. It may find possible applications in the industrial fields and in biotechnological processes.
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1. Introduction:

Microorganisms can synthesize three types of polysaccharides defined by their cellular location; 1) the intracellular polysaccharides, known as the storage form of carbon; 2) the cell wall polysaccharides such as peptidoglycan or teichoic acids and; 3) the extracellular forms, 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**). Furthermore, presence of EPSs favors the interaction between supports and bacteria, which is involved in the appearance of biofilm (**Gruter *et al.* 1993**). EPSs are often thought to have a protective function for microbial cells; the ability of a microorganism to surround itself in a highly viscous layer may provide a protection against desiccation or phage attack (**Gruzdev *et al.* 2011**). Cells buried within a polymer matrix may be inaccessible to antimicrobial agents such as antibiotics and resist the penetration of toxic metal ions to the cells (**Jiang *et al.* 2011**). EPSs can also enable free-living bacteria to adhere to and colonize solid surface where nutrients accumulate. Some EPSs exhibit flocculating properties (**Wang *et al.* 2011**). Flocculation is the term used to describe a property expressed by some microorganisms, when they spontaneously aggregate to form flocs with sediments in the culture medium. Microbial bioflocculants have received increasing scientific and technical attention because of their special advantages such as safety, strong effect, biodegradability, and biocompatibility. EPS bioflocculants are not only nontoxic but also their degradation intermediates are not secondary pollutants (**Zhang *et al.* 2002**).

Various environmental signals 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 a protective function allowing adaptation to stresses, regardless of the nature of the stress factor. A proteomic study in *Streptomyces coelicolor* was performed to

identify the relationship between proteins expression and environmental stresses, and proteins expression at each phase of cell growth. It was demonstrated that almost all of the shock-related proteins were found in the transient phase just before the stationary phase, implying that various shocks induces the proteins responsible for the initiation of the stationary phase. Based on these findings, it can be deduced that certain types of environmental shock could cause a premature initiation of secondary phase and thus an earlier initiation of secondary metabolites production **(Kim *et al*. 2008).**

Fungal species belonging to the phylum Zygomycota are characterized by sexual reproduction via zygospores, or asexual reproduction by uni- to multi-sporangia. The Zygomycetes 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 (**Mandanas 2011**). 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. Furthermore, *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 1994)**, and oleanolic acid derivatives (**Capel *et al*. 2011**).

The fungus *M.rouxii* was found to produce a biopolymer flocculant during growth at pH 3.5, using beet-molasses as a carbon source. This feature led us to study the response of the fungus when exposed to an acidic pH-shock. The present study dealt with the production of an EPS bioflocculant by *M.rouxii* when exposed to some abiotic stressors. The resultant EPS was also tested to characterize some of its properties.

2. Materials and Methods

Microorganism and growth conditions

For production of the EPS, *M.rouxii* was grown in a minimal medium of beet molasses (BM) containing (g/L): 30.0 beet-molasses as a carbon source and, 1.5 $(NH_4)_2SO_4$ as a nitrogen source. For comparison, two other medium were used, potato-dextrose broth (PDB) or glucose enriched medium (GEM). The later containing (g/L): glucose, 20; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; $ZnSO_4.7H_2O$, 1.8 mg; $FeSO_4.7H_2O$, 1.0; $MnSO_4$, 0.3 mg; $CuSO_4.5H_2O$, 0.4 mg (**Bartniki-Garcia and Nickerson, 1962**). 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^oC for 5 days. All flasks were inoculated with a pre 7-days culture of *M.rouxii*, grown on potato dextrose agar.

Detection of the exopolysaccharide

The produced EPS was expressed by detection of the flocculating activity (FA) using charcoal as the test suspension as described by (**Zhang *et al.* (2002**).

Influence of stress conditions

The fungus was exposed to stress condition such as oxygen tension, osmolarity, extremes of pH values (3.5 and 10.0), elevated temperature (50-70^oC), drying at room temperature for 2 months, or freezing at refrigerator conditions (**Pirog *et al.* 1997, Petronella *et al.* 1999**).To study the protective function of the EPS against the extremes of pH, the cultures was alkalized to pH 10.0 with a 6% NaOH solution or acidified to 3.5 with a 6% HCl solution and then the FA was determined during growth phase. To elucidate the role of EPS in protection of cells from the effect of high temperature, the cultures was heated in a water bath to 50, 60, or 70^oC for 15 min, then the FA was determined during growth of the cells under shaking conditions (**Pirog *et al.* 1997**). The role of the EPS in protection of cells against drying was studied by keeping 5 ml of a culture in sterile petri dish at room temperature (for 2-months) until it completely dried out, after which the dried cells were suspended in 3-ml sterilized distilled water, cultured under shaking conditions (130 rpm), and then the FA was determined. The ability of the EPS to protect the cells against osmolarity, each of NaCl, sucrose, or glycerol was used at different concentrations (**Petronella *et al.* 1999**). To investigate the ability of the EPS to protect fungal cells from low temperatures, a culture was kept in a deep freezer for 4 days, and then was tested for EPS formation after thawing the frozen cell suspension. The whole procedure of freezing, thawing, and detection of the EPS was repeated.

Stability of the exopolysaccharide

Degradation of the produced EPS by enzymes and detection of the liberated reducing sugars was performed by the DNS method (**Miller, 1959**).

Chemical analysis of the exopolysaccharide

Protein content was detected by the method of Lowry (**Lowry *et al.* 1951**). The content of amino-sugars in the EPS was measured by the Elson-Morgan method (**Chaplin and Kennedy**, **1986**),

using glucosamine as the standard. The average molecular weight of the EPS in relation to the viscosity was calculated according to the method of **Il'ina *et al.* (2001**). The viscous EPS 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 EPS, chemical composition and mineral content of beet-molasses, and atomic absorption were detected by the "Central Services Laboratory", National Research Center.

3. Results

Growth of the fungus under normal conditions

M.rouxii grew effectively in minimal medium containing beet-molasses (without any pretreatment). The chemical composition and element content of beet-molasses are represented in Table (1). As shown, beet-molasses contain high concentration of Na^+ and K^+. In addition, beet-molasses contain also high concentrations of Fe^{2+}, Zn^{2+}, as well as the total acidity. The biological function of an EPS should be studied on model system closely resembling the natural environment of the producer. In particular, the protective function should be investigated under different growth conditions, i.e. optimum, non optimum, and extreme. Low production of the EPS by *M. rouxii,* and low viscosity of culture broth were observed under normal conditions (1-2 % beet-molasses, pH 6.0, and at 28°C), throughout the lag to the stationary phases. As shown in Fig. (1), the organism exhibited normal growth up to the stationary phase, the amount of EPS produced (expressed as FA %) was low and reached its maximum in the stationary phase (96 hrs of growth), and the pH of the culture broth decreased by time. Worthy mention is that, cells of *M. rouxii* form pellets during normal growth, except under unfavorable conditions; the cells extremely aggregated and form flocs due to the secretion of EPS to protect the fungal cells.

Influence of stress conditions

A key parameter in submerged cultures is oxygen tension (0T) which is significantly influences cell growth and the fermentation process**.** Experiments for studying the effect of OT on formation of the EPS were performed in 250-ml conical flasks containing different volumes of culture broth, using 3 and 4% beet-molasses. Results revealed that, reduction of oxygen increased the lag phase, decreased the growth rate, but did not enhance formation of the EPS (Table 2). On other hand, addition of water activity-reducing components, such as glycerol, NaCl, and sucrose affected the growth of cells but did not enhance formation of the EPS (Table 2). Normal growth of *M. rouxii* was observed at pH values of 5.0-7.5. Formation of the EPS gradually increased and reached a maximum (87%) at pH 10.0 (Table 2). However, when the initial pH of the growth culture was adjusted to 3.5 the FA reached 99% (Table 2). Elevation of temperature to 50°C did not enhance formation of the EPS. At 60°C, the FA reached 73% with increasing the viscosity of the culture broth (Table 2). Heating a culture in any growth phase to 70°C increased the viscosity and EPS production; the FA reached 92% (Table 2). A two–months drying of cells moderately increased the EPS production; the FA reached 48 % (Table 2). Freezing of *M. rouxii* cells contributed to the formation of EPS by 61%. Effect of medium components on production of the EPS, at pH 3.5, revealed that the EPS was increasingly produced by *M. rouxii* when grown in BM medium rather than in GEM or PDB medium (Fig. 2).

Table 1. Chemical composition and mineral content of beet-molasses

Parameter	Concentration
Chemical composition:	(g/100g)
Moisture	12.43
Total acidity	49.70
Reducing sugars	22.00
Non-reducing sugars (sucrose)	25.50
Ash	6.40
N2	1.83
Minerals and Trace elements:	(mg/100g)
Mn^{2+}	2.46
Fe^{2+}	7.80
Zn^{2+}	12.60
Cu^{2+}	2.50
Ca^{2+}	11.50
Mg^{2+}	37.40
Na^+	986.80
K^+	201.00

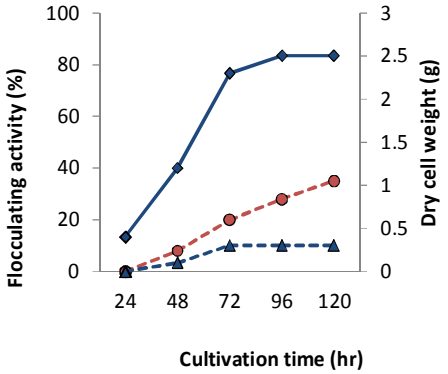


Fig. 1. Growth of *Mucor rouxii* (♦) in beet-molasses medium (2%), the flocculating activity, % (●) of culture broth, and the viscosity (▲) during normal growth.

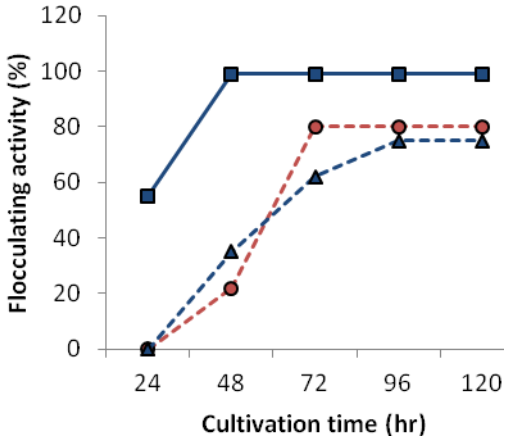


Fig. 2. Production of the exopolysaccharide by *Mucor rouxii* during growth in different media: beet-molasses, 3% (■),glucose-enriched medium (●) or potato-dextrose broth (▲) at pH 3.5.

Table 2. Effect of some abiotic stresses on flocculating activity*, viscosity and dry cell weight of *Mucor rouxii*,.

Factor	DCW**	FA (%)	Viscosity (*mPa*.S)
Oxygen tension	1.48	0	0
Osmotic pressure	1.41	0	0
pH value:			
3.5	0.75	99	2.44
10	0.99	87	2.20
Temperature, °C:			
50	0.95	30	0.48
60	0.45	73	1.12
70	0.28	92	1.44
Desiccation:			
2-months	1.32	48	0.63
Freezing	1.19	61	0.75

*Production of the exopolysaccharide was expressed as flocculating activity (FA, %). **DCW=dry cell weight (g) after 48 hrs of growth.

Under optimized culture conditions (3% beet-molasses, 0.15 % $(NH_4)_2SO_4$, pH 3.5 at 28°C for 48 hrs growth), 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.

Distribution of the exopolysaccharide

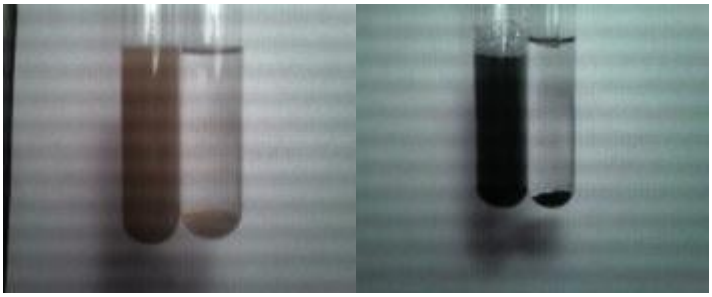
To investigate the distribution of FA of the EPS during the recovery procedures, each of the FA of the culture broth, cell–free supernatant, and the supernatant after ethanol precipitation (for recovery of the EPS) was detected. The FA of culture broth was almost the same as that of its cell-free supernatant, whereas the supernatant after ethanol precipitation showed no flocculating activity.

Some properties of the exopolysaccharide

The flocculating activity property

The crude EPS produced by *M. rouxii* was found to possess good FA property. The particles of soil or charcoal were effectively aggregated and precipitated by addition of the EPS through 5 min (Fig. 3, a and b). Using charcoal as the suspended particles (Fig. 3,a), the FA of the culture supernatant reached 99%, at a concentration of 1% (v/v) without addition of cations. 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 (Fig. 3b and Fig. 4). However, addition of trivalent cations such as Al^{3+} or Fe^{3+}, completely inhibited the FA (Fig. 4). On other hand, it was found that higher or lower dosage of the EPS bioflocculant resulted in low

flocculating efficiency. Maximum FA was observed at a concentration of 10 or 2 ml/L of the crude and purified EPS bioflocculant, respectively (data not shown).



(a) (b)

Fig. 3. Efficacy of the crude exopolysaccharide produced by *Mucor rouxii* in aggregation and precipitation of soil (a) or charcoal (b) particles, after 5 min. The figures: (a) and (b) showed the precipitating action (right image) compare with the control (lift image).

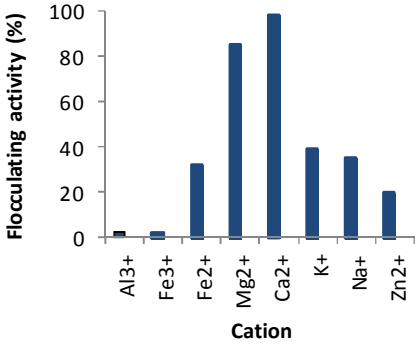


Fig. 4. Effect of cations on the flocculating activity of the crude exopolysaccharide bioflocculant. The indicated cations were used as chlorides; 0.25 ml of 8 mM cation was added to a reaction mixture containing soil particles.

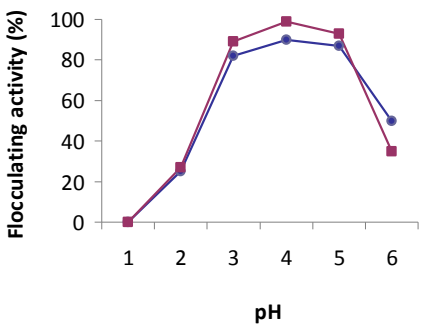


Fig. 5. Effect of pH on the flocculating activity of the crude (●) and purified (■) exopolysaccharide bioflocculant.

Effect of pH

The effect of pH 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 EPS bioflocculant, was adjusted with HCl or NaOH and then the FA was measured. As shown in Fig. (5), high FA was detected in an acidic pH range of 3.0-5.0 with a maximum at pH 4.0. These results revealed that the EPS produced by *M. rouxii* is a novel acidic polysaccharide.

Chelation of heavy metals

The produced EPS bioflocculant exhibited flocculating abilities for sorption of cations involved in beet-molasses; it removed 89, 83, and 78 % of Mn^{2+}, Fe^{2+}, and Zn^{2+}, respectively from beet-molasses (data not shown). The efficiency of the EPS bioflocculant for chelating some heavy metals (Pb^{2+}, Hg^{2+}, Zn^{2+}, 1 ppm) was also examined. The percentage of heavy metal removal by the EPS bioflocculant (10 ml/L) was 92, 89, and 78% for Pb^{2+}, Hg^{2+}, and Zn^{2+}, respectively (Fig. 6).

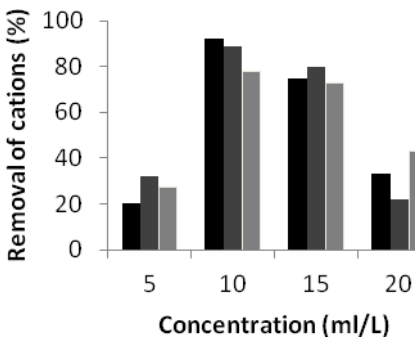


Fig.6. Effect of the bioflocculant concentration on removal of: (■) PB^{2+}, (■) Hg^{2+}, or (■) Zn^{2+}, with soil particles.

Stability

It would be of interest to study the stability of the purified EPS against degradation by enzymes. In the present study, a variety of purified enzymes (chitinase, chitosanase, and *B*, 1-4 glucanase) produced by *Bacillus alvei* NRC-14 were tested for degradation of the EPS, individually, or in combination. Results revealed that: firstly, individual use of chitosanase or *B*-,1-4 glucanase resulted in an obvious degradation of the EPS as indicated by detection of reducing sugars (Fig. 7). Secondly, an efficient degradation of the EPS was achieved when a mixture of these enzymes was used; large amounts of liberated reducing sugars were detected (Fig. 7). Interestingly, the crude EPS was also stable against degradation by enzymes. On other hand, the heat-stability of the EPS from *M. rouxii* revealed that it is a heat-stable polysaccharide; it remained about 99 % of its FA after heating at 100°C for 20 min (data not shown).

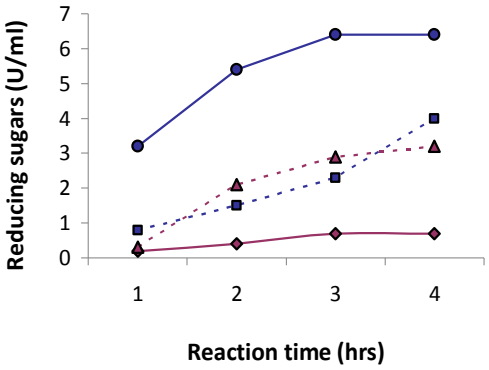


Fig.7. Degradation of the purified exopolysaccharide by purified: (♦) chitinase, (■) chitosanase, (▲) *B,* 1-4 glucanase, or (●) a mixture of all.

Composition and molecular weight

The biopolymer produced by *M. rouxii* is an exopolysaccharide comprised carbohydrates (96%) and protein (4%). The molecular weight of the EPS 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. 8).

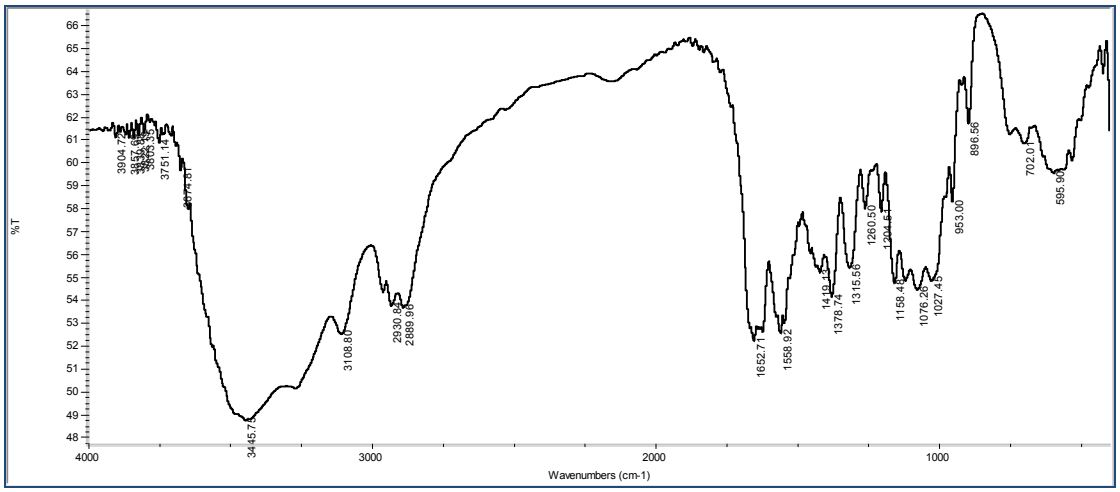


Fig. 8. IR-spectra of the exopolysacchride produced by *Mucor rouxii.*

4. Discussion

The concept of stress can be defined as a combination between stressor and stress reaction (stress = stressor + stress reaction; a stressor is what triggers the stress reaction). 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**). 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). It was concluded that an acidic pH-shock may induce the formation of an EPS by the fungus to protect the cells. In the present study, we investigated the response of the fungus to produce extracellular EPS when exposed to abiotic stress. Results revealed that, low formation of the EPS by *M. rouxii* was observed under normal conditions. When the fungus was exposed to abiotic stress such as oxygen tension or osmolarity, low formation of the EPS was observed. Comparable results were reported previously **(De Vuyst et al. 1998, Petronella and Jeroen 1999**). It seems that oxygen tension or low-water activity could not be used as tools to increase an exopolysaccharide production. Exposure of the fungus cells to drying or freezing increased the formation of the EPS to some extent. However, elevated temperature to 70^{o}C triggered the EPS formation by 92%. **Pirog et al. (1997)** reported that, elevation of temperature to 70°C led to a complete loss of cell viability of an *Acintobacter* sp. strain, with higher viscosity of the EPS solution. The amount of the synthesized EPS increased, especially in the exponential phase and early stationary phase. This may also be explained by the presence of other protective mechanisms which may exist in the culture liquid, such as enzymes or other polymers, contributing to bacterial resistance during the exponential and stationary phases (**Gruzdev *et al.* 2011**). Thus, the acidic pH shock and elevation of temperature (70^{o}C)) were the strongest factors for the EPS formation to protect fungal cells. Exposure of the fungus cells to an acidic pH shock resulted in triggering the formation of EPS. The FA reached 99, 80, and 75% in BM, GEM, and PDB media, after 48, 72, and 96 hrs, respectively. Higher formation of the EPS was observed in BM medium rather than GEM or PDB medium (Fig. 2). It seems that, high concentrations of Na^{+}, K^{+}, total acidity as well as presence of $Fe^{2+}$and Zn^{2+}in beet-molasses could be additional stressors resulted in triggering the production of the EPS as a form of self-protection during fungal growth. Quick formation of the EPS by *M. rouxii* after 48 hrs of growth at pH 3.5 in BM medium may confirm an assumption that the chemical composition of beet-molasses may be another factor for enhancing formation of the EPS.

It is well established that, all living organisms, i.e. bacteria, fungi, plants, animals, and humans synthesize specific proteins in response to temperature elevation, known as heat shock proteins (HSPs). Overproduction of HSPs renders microbial cells resistant to the abiotic stress, especially in the exponential and early-stationary growth phases of a microorganism (**Vorob'eva, 2004**). ***Heat-shock*** response by microorganisms is not limited to the changes in temperature but also serve general protective functions, allowing adaptation to stress and starvation, regardless of the nature of the stress factor (**Kim *et al.* 2008**). The HSPs include chaperones and proteases that are, presumably, essential for overcoming changes that involve protein denaturation. Denatured proteins that are produced under stress conditions may aggregate, exerting toxic effects in the microbial cells (**Ron *et al.* 2000**). So, microorganisms are equipped with a defense system, which is based on their ability to degrade defected proteins. Defected proteins (denatured, inactive, or unstable proteins) are recognized by the proteases or the chaperone complex (**Vorob'eva, 2004**). Such defected proteins are either cleaved into small peptides by proteases and eliminated, or recovered into functional proteins by the chaperone complex. **Kim *et al.* (2008)** have reported that, the biosynthesis of actinorhodin (a product produced by *Streptomyces coelicolor*) increased by an acidic ***pH-shock*** which was considered to be one of the strongest stressors to influence shock-related proteins and trigger the formation of actinorhodin. Enhancement of kasugamycin production by *Streptomyces aureofaceins* due to an acidic pH-shock was also reported **(Kim et al. 2000).**

The EPS from *M. rouxii* exhibited good FA, using soil or charcoal particles. However, during the flocculation of charcoal suspension, the flocculating

rate was high without addition of cations, but Ca^{2+} and Mg^{2+} were needed when the EPS flocculant was used to flocculate soil particles. This may be due to the presence of more negative charges on soil particles **(Deng et al. 2003)**; the presence of cation(s) affects the FA by neutralizing the residual negative charges of functional groups by forming bridges between particles **(Salehizadeh et al. 2000).** Other polysaccharide bioflocculants 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 bioflocculant from *Bacillus* sp. AS-101 (**Salehizadeh et al. 2000**). On other hand, the acidic EPS bioflocculant from *M. rouxii* exhibited efficiency for biosorption of heavy metals, and this may elucidate the ability of the fungus to persist in high concentrations of beet-molasses and reveal its capability to synthesize an EPS as a form of self-protection. Biosorption of heavy metals by novel acidic polysaccharide bioflocculants from microorganisms have been reported **(Lin and Harichund 2011).** Generally, EPS biopolymers 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 2011).**

The MW of the EPS produced by *M. rouxii* was found to be 1.78 x 10^6 Da. The MW and functional groups in the molecular chains are the 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, leading to the formation of three-dimensional flocs that are capable of settling fast **(Zhang *et al.* 2002).** Higher or lower dosage of the EPS 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 EPS produced by *M. rouxii* is highly stable; it requires a complex of enzyme preparation which could act synergistically to degrade several bonds in the carbon chain. It is well known that most enzymes that capable of degrading polysaccharides are belonging to the glycosidase family (amylase, cellulase, chitinase, chitosanase, etc). So, unlike most microbial polysaccharides, the EPS produced by *M. rouxii* is a biologically highly stable polymer. The protection of cells may, probably, be due to the stability of this EPS which is highly resistant to biodegradation. The result of this study is in accordance with previously observations reported by **Pirog *et al.* (1997**) for an EPS from *Acinetobacter* sp. They explained that an enzyme alone may only be specific for a certain chemical bond in the carbon chain of an EPS, and that a complete degradation of an EPS, probably, requires a complex of enzyme preparation or series of enzymes produced by mixed cultures.

The spectrum of the purified EPS 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 –NHCOCH3. A weak band could be observed at 1419 cm^{-1}, which 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 EPS from *M. rouxii* contains important functional groups such as carboxyl, hydroxyl, and urinate. 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)**. Presence of urinate assists metal uptake and allows for adhesion of microorganisms to surfaces, whereas carboxylate groups act as non-specific ion-exchange material which may convey chelating property **(Lin and Haeichund 2011).**

To date, various biopolymers have been produced from different microorganisms. The produced 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 byproduct of sugarcane industry to reduce the production costs.

Conclusion:

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 EPS bioflocculant by the fungus *M. rouxii*.

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

3) The extracellular polysaccharide was found to be synthesized by the fungus due to exposure to abiotic stress. The acidic pH-shock was considered to be the strongest stressor to induce the biosynthesis of this EPS as a form of self-protection by the fungus.

4) The produced EPS possesses good flocculating activity, highly stable against biodegradation by enzymes, and exhibited potentiality for removal of heavy metals. It may find possible application as a biopolymer for biotechnological processes. Further studies are in progress to realize its industrial utilization.

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