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Response of *Kluyveromyces lactis* to Sodium Chloride Salt Stress and the Possibility of Enhanced Lipids Production

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Microbial oils might become one of the important and effective feed-stocks for biodiesel production in the near future. The use of these oils is currently hot topics in research in order to reduce production costs associated with the fermentation process, which is a crucial factor to increase economic feasibility. An important way to reduce processing costs is the use of wastes as carbon sources. The growth of *K. lactis* gradually increase as NaCl increase in the growth medium up to 6% (w/v). The highest percentage of increase was 178.38% on 2% NaCl containing medium. While glycerol and total lipids increased with 133.61 and about 56 % as NaCl increased in up to 8%. However, phospholipids significantly increased (P < 0.001) but neutral lipids and sterol decreased as NaCl increased in the growth medium. Stearic (18:0) and behenic (22:0) fatty acids increased while oleic (18:1) and linolenic (18:3) decreased. On the other hand, palmetic (16:0), linoleic (18:2), arachidic (20:0) and lignocaric (24:0) fatty acids appeared in presence of NaCl in the growth medium.

Keywords: *Kluyveromyces lactis*, Stress, Sodium chloride, Lipids.

Yeasts and fungi in general are more endure salt stress other than bacteria, although microorganisms differ in their tolerance to this stress [1].

Salt-in strategy and the "compatible-solute" strategy are the two know mechanisms for organism's response to salt stress. The first strategy is use by bacteria[2] . However, "compatible solute" strategy was defined as solutes when present at high concentration permit the enzymes to work properly[3]. "Compatible-solutes" are accumulated by the organism to be important carbon and nitrogen sources. These solutes include polyols such as glycerol, mannitol, sugars, or sugar derivatives; betaines and amino acids, including proline, glutamate, and glutamine, as reported by Grant [4], additionally glycerol has since been reported as the major internal osmolyte in four xerophilic fungi by [5,6].

Membrane lipids play a central role in mediating membrane functions by acting as physical barriers to diffusion of electrolytes and are solvent for a variety of constituents and conformational stabilizers of membrane proteins[7]. The concept of membrane fluidity is often used to describe the relative motional freedom of membrane lipids[8]. Several factors are involved in the maintenance of proper membrane fluidity: the type of fatty acyl chains (their length and unsaturation), the amount of sterols and a lesser extent, the nature of the polar phospholipids head-groups [8]. Matsakas et al. [9] used of sweet sorghum at high solid concentrations by *Rhodosporidium toruloides*; the addition of enzymes permitted liquid fermentation at high substrate concentration

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were enhanced lipid production by 85.1% and 15.9% when dried stalks or stalk sorghum juice were used, respectively. EL-Mougith [10] reported that total lipids, phospholipids, free fatty acids, free sterol, and the saturated fatty acids in fungal mycelia of *Penicillium notatum*, *P. chrysogenum* and *A. flavus* increased as the NaCl concentration increased. Salt stress induced important changes in phospholipids and fatty acids composition of *P. corylophilum* and *Halobacterium halobium*[11]. Andreishcheva *et al.* [12] found that salt stress was accompanied by an increase in the intracellular level of glycerol and free amino acids as well as by changes in lipids and fatty acids composition. The aim of the present work is to investigate lipids formations in response to salt stress in *K. lactis*. Lipids produced by microorganisms can be used as potential feedstock for biodiesel production. These oils have several advantages: Short life-cycle, less labour required, less affected by season and climate, and easier to scale up[13].

MATERIALS AND METHODS

Yeast strain and growing

The strain *Kluyveromyces lactis* IFO1267 (a prototroph [K2K1]) that was kindly provided by Dr MJR Stark, the University of Dundee, Dundee, England.

YEPD broth (1.0% yeast extract, 2.0% peptone, 2.0% glucose, 0.1M citrate-phosphate buffer pH 4.5) was used for growing *Klyuveromyces lactis*. YEPD-agar (YEPD containing the same components of broth in addition to 2.0% agar), this medium was supplemented by different NaCl concentrations (0.0% "control", 2%, 4%, 6% and 8% (w/v), for each concentration triplicates were used. After sterilization by autoclaving at 121ÚC for 20 min, the flasks were inoculated after cooling with 1.0 ml of 10^7 cells suspension prepared from 24 hours-old cultures grown on YEPD liquid medium that shaked at 130 rpm on a rotary shaker for different specified time period.

Analytical techniques

Glycerol estimation

Glycerol was estimated according to the method described and adapted by Mansour [14]. Ten ml of clarified soluble sugar extract were mixed with 5 ml of 7.5% aqueous solution of potassium bichromate and 30 ml of 50% suphuric acid. The mixture was mixed and boiled in water bath for 15 min, cooled, and made up to 50 ml by distilled water. The developed color was measured in photoelectric colorimeter at 350 nm using glycerol as standard.

Lipid analysis

The fresh yeast cells were harvested after ten days of growth, that were washed with distilled water and fixed by immersing in boiling distilled water for 5 min. Total lipids were extracted as described by Bligh and Dyer[15] using chloroform/ methanol (2/1, v/v) for 2 hr. twice, then the two extracts were combined. After evaporation of the organic solvents, the residue was dissolved in chloroform, washed with NaCl solution (0.9%) and dried over anhydrous $NaSO_4$. Phospholipids were determined by phosphorous assay according to Rouser *et al.*[16]. Known volumes of lipid samples were transferred into clean glass tubes and the solvent "chloroform" was completely evaporated. 0.65 ml of 70% perchloric acid was added and then the tubes were placed in hot sand bath for about 30 min or until complete clearness. After cooling 3.3 ml distilled water, 0.5 ml of ammonium molybdate solution then 0.5 ml of ascorbic acid solution were added to each tube with shaking after each addition. The tubes were placed in a boiling water bath for 5 min. The absorbance of cooled samples was read at 800 nm (Milton Roy, Spectronic 20D) using KH_2PO_4 solution as standard.

Estimation of fatty acids and sterols

Fatty acids and sterols were separated by saponification, where 1 ml of lipid extract was evaporated under vacuum at room temperature, 4ml of absolute ethyl alcohol was added followed by addition of 0.4 ml KOH solution (33%) for hydrolysis at 90ºC for 4 hr. in water bath. All tubes were cooled and equal volume of distilled water was added. Five ml of hexane were then added and the system was shaken in a separating funnel. The upper layer containing sterol was evaporated and the residue represented the free sterol. The lower layer which contained fatty acids were neutralized with HCl and extracted as it was done for sterol by hexane. The methylation of fatty acids was done as described by Weete *et al.*[17] where two ml of borontrifloride were added to the dried residue of fatty acids. The tubes were boiled for 2-3 min in a water bath, cooled in ice bath and 2 ml of distilled

water were added. A suitable volume of hexane was added, the hexane layer which contained fatty acid methyl ester was evaporated and the residue was suspended in a known volume of chloroform for estimation of total fatty acids by HPLC system (USAID, HPHELETT, serial, FERIES1050, located in The Agricultural Center, Plant Diseases Institute, Cairo, Egypt) according to the method of [18]. Total sterols were estimated by the Libermann-Burchard reaction as described by Hosono and Aida[19]. Five ml of chloroform suspension containing sterols were mixed with 2 ml acetic anhydride/ sulphuric acid (4:1, v/v) and kept for 15 min at 18ºC. The sterol content was measured immediately by measuring the absorbance at 625 nm (Milton Roy, Spectronic 20D) using cholesterol as standard.

RESULTS

Effect on growth

Table (1) represents the growth of *K.lactis* (mg dry weight/50 ml medium) in the presence of different concentrations of NaCl (0.0% ‘’control”, 2%, 4%, 6% and 8%). The fungal biomass increased as NaCl concentration in the growth medium increased and the percentages of these increases were 300.00%, 233% and 143.00% at 2%, 4% and 6% NaCl, respectively. Then the growth was reduced by 53.3% at 8% NaCl as compared to control.

Effect on glycerol

There is a gradual increasing of the glycerol biomass content (mg/g dry weight) of *K.lactis* grown at 2%, 4%, 6% and 8% NaCl and the percentages of these increases were 61.34%, 81.4%, 112.4% and 133.61%, respectively as compared to that of control (Table 2).

Effect on lipid composition

There is a gradual increasing of total lipids and phospholipids of *K.lactis* biomass grown at 2%, 4%, 6% and 8% NaCl and the percentages of these increases were 159, 173, 205 and 215% for total lipids, 321.85, 426.00, 557.4 and 614.80% for phospholipids, respectively as compared to that of control (Table 3). On the contrary, there is a gradual decrease in neutral lipids and sterol of *K. lactis* biomass grown at 2,4,6 and 8% NaCl and the percentages of these decreases were 8, 20, 21 and 28%, for natural lipids and 3.50, 15.00,27.00 and 61.00% respectively as compared to that of control. It was also found that, the percentages of increases of phospholipids were very high, while the percentages of decreases of neutral lipids were relatively low, when compared with control.

Effect on fatty acids composition

The data in Table (4) showed that, in non-treated fungal biomass oleic (C18:1) was the most abundant fatty acid, while the most abundant fatty acids in NaCl-treated fungal biomass grown at 2, 4, 6 and 8% NaCl were palmitoleic (C16:1), and stearic (C18:0), respectively. Some fatty acids were detected only in yeast biomass grown at higher concentrations of sodium chloride (6 and 8% NaCl), these fatty acids were palmitic (C16:0), linoleic (C18:2), arachidic (C20:0) and lignoceric (C24:0).

Table 1. Effect of NaCl concentrations on the growth (as mg/50ml medium) of *K. lactis* on YEPD broth medium , pH 6 at 28°C for 72 hrs

Dry weight “mg/ 100ml medium”	NaCl (% w/v)
3.0±0.2	0.0
9.0±0.2**	2
7.0±0.5**	4
4.3±0.3**	6
1.6±0.1***	8

* Significant, * p < 0.05, ** p < 0.01, *** p < 0.001

Table 2. Influence of NaCl concentrations on glycerol as mg/g dry weight of *K. lactis* on YEPD broth medium, pH 6 at 28°C for maximum growth

NaCl %	Glycerol (mg/g dry weight)
0	6.10±0.57
2	10.00±0.89*
4	11.50±1.00**
6	13.00±1.02**
8	14.00±1.16***

* Significant, * p < 0.05, ** p < 0.01, *** p < 0.001

DISCUSSION

The results of this investigation revealed that *K. lactis* is a halophilic yeast due to its ability to grow at these high concentrations of sodium chloride.

Several studies have shown that osmotically stressed yeasts and filamentous fungi accumulate polyols or sugars during their growth to equilibrate internal and external osmotic pressure[20,21]. Glycerol is consistently reported to be the main cytoplasmic polyol produced in fungi under conditions of increased solute-associated water stress [22, 23]. In this study, it was found that glycerol content of *K. lactis* increased as the NaCl concentration increased as compared to that of control. This may be due to activation of certain enzymes involved in glycerol production pathway such as glycerol 3-phosphate dehydrogenase and glycerol 3-phosphatase[22]. Schuurink *et al*.[24] and deVries *et al*.[25] demonstrated the constitutive production of $NADP^+$-dependent glycerol dehydrogenase in *Aspergillus nidulans* and *A. niger* as response to salt stress. Later, the osmotic adaptation of the halophilic fungus *Hortaea werneckii* was studied by [26] and it was found that glycerol was the major compatible solute in actively growing *H. werneckii*.

Changes in the membrane composition and properties represent an important factor in the adaptation to high salt concentration[27]. Membrane lipids are important in controlling membrane fluidity[8]. Sodium chloride induced only quantitative changes in lipid components. This may be due to activation of certain enzymes and deactivation of others (Elwan *et al*.[28] and Kassem *et al*.[29]. Neutral lipids of *K. lactis* decreased with increasing NaCl concentration in the growth medium. EL-Moughith[10] reported that neutral lipids of *P. notatum, P. chrysogenum* and *A. flavus*

Table 3. Effect of NaCl concentrations on lipid metabolism of *K. lact*is on YEPD broth medium, pH 6 at 28ÚC for maximum growth

NaCl (%)	Total lipids	p	Lipid fractions "as mg/g dry weight" Phospholipids	p	Neutral lipids	p	Total sterol	p
0	25.80±2.00		5.40±1.51		15.70±1.62		4.70±0.38	
2	36.32±3.66	S**	17.38±4.19	S**	14.40±1.40	NS	4.54±0.30	S**
4	39.6±4.22	S**	23.00±3.02	S**	12.60±1.00	S*	4.0±0.49	S**
6	45.93±4.60	S***	30.10±2.78	S***	12.40±1.10	S*	3.43±0.28	S**
8	46.37±14.01	S**	33.20±5.31	S***	11.34±1.00	NS	1.83±0.88	S**

NS: Non significant
S: Significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 4. Fatty acid composition "as % of total fatty acids" of *K. lacti*s grown at different NaCl concentrations on YEPD broth medium, pH 6 at 28ÚC for maximum growth

NaCl%	Fatty acids "as % of total fatty acids" C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C24:0
0	ND	25.87	40.66	50.00	ND	2.00	ND	3.52	ND
2	ND	ND	44.00	12.00	ND	1.58	ND	4.80	ND
4	ND	33.18	46.22	11.30	ND	1.41	ND	ND	ND
6	4.59	12.35	49.11	11.50	2.30	1.29	4.93	4.85	4.88
8	5.98	12.19	54.00	10.80	1.16	1.00	8.48	4.9	4.61

Unsaturation index = $\frac{1(\%\ \text{monoenes}) + 2(\%\ \text{dienes}) + 3(\%\ \text{trienes})}{100}$ mol as defined by Kates and Hagen (1964)

ND = Fatty acid not detected

decreased with increasing NaCl concentration in the growth medium. In contrast, neutral lipids of *Cladosporium cladosporioides* mycelium increased due to salt stress[30]. Total phospholipids of *K. lactis* were increased with increasing NaCl concentration in the growth medium. These results are in contrast with those found by EL-Moughith[30]. He mentioned that total phospholipids of mycelium and plasma membrane of *C. cladosporioides* decreased at high salinity. However, similar observations were reported that total phospholipids in *P. corylophillium*, *Halobacterium halobium* and *A. ochraceus* had increased in the presence of NaCl in the growth medium (Hefnawy *et al*.[11] and Metwally and Hefnawy[31]).

Sterols generally decrease the fluidity of the lipid phase and natural membranes by reducing lipid acyl chain mobility[32]. The total sterol of the tested fungus was increased as response to salt stress. The obtained results don't agree with the previous results in the halotolerant yeasts *Debaryomyces hansenii* (Tunblad-Johansson et al.[32] and *Yarrawia lipolytica* (Andreishcheva et al.[12] in which an increase in NaCl concentration caused a decrease in sterols. Similar results were the halophilic melanized yeast-like fungus Hortaea werneckii (Turk et al.[33]) showed an increase in total sterols. Also, changes in the composition of fatty acyl chains, such as unsaturation, length and branching are thought to affect membrane fluidity[8]. In this work when *K. lactis* grown at different NaCl concentrations, the salt stress affect the composition of fatty acids, although these changes were not the same in all treatments. It was also appeared that salt stress induced qualitative and quantitative changed in the total fungus fatty acids which may play an important role in the unsaturation index and the selective permeability of the membranes the changes of the membrane fluidity. The study of *Alternaria chlamydospora* (Mulder *et al*.[34]) and *Hortaea werneckii* (Turk *et al*.[33]) showed an increase in unsaturation of fatty acids as NaCl concentration increased.

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