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Efficiency of Moderately Halophilic *Bacillus* spp. Isolated From Hypersaline Environments In Producing Levansucrase

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ABSTRACT

Background: Moderately halophilic bacterial strains were isolated and identified four species belong to*Bacillus*spp. fromhypersaline environments (Sawa Lake), then determined their tolerating on nutrient agar supplemented with variousconcentrations of NaCl and sucrose ranges (10-200)gm/l and ability to produce a slimy mucus when grown in tryptonesucrose agar medium with sucrose (20gm/l), as carbon source. Results showed that *Bacillus* isolates were grown in concentrations of NaCl and sucrose up to 140gm/l and 200gm/l, respectively. Biomass value for (Saw 2) isolate wasincreased gradually during a fermentation media supplemented with mixture of (NaCl+Sucrose) (10-100)gm/l, after incubated for 48h at 30ºC and produced levansucrase at concentrations (1-10)gm/100ml of (NaCl+Sucrose) with activity(462.08, 463.33, 460.83, 460.83, 455.41, 453.75, 452.97, 454.58, 454.16 and 452.07)U/ml and descending in quantity of enzyme activity (439.37, 450.41, 431.66, 422.29, 381.04, 361.87, 312.29, 315, 276.25 and 251.87)U/ml when added sucrose to fermentation media more than 4gm/100ml.

Key words: Halophilic bacteria, Osmophilic bacteria, Levansucrase production.

INTRODUCTION

Moderately halophilic species of bacteria belong to *Bacillaceae* and *Micrococcaceae*, such as *Paracoccus halodenitrificans* and *Micrococcus* spp. involved in a wide diversity of natural habitats and flourish best in media containing (3-15)% (W/V) NaCl (Kushner and Kamekura, 1988). They produce salt stable enzymes due to their ability to carry out catalysis under high salinity (Karan *et al.*, 2012).

There are different strategies utilized by halophilic microorganisms to adjust their cytoplasm osmotically with their medium, includes aggregation of the molar concentrations of salts, similar to potassium and chloride, which requires broad adjustment of the intracellular enzymatic apparatus, as the proteins should maintain their structure and activity at high concentrations of salt (Lanyi, 1974).

Water availability [Water activity (a_w)] resolves both the vitality and practically of living systems. The most of microbes cannot multiply below 0.900 a_w (Manzoni *et al.*, 2012; Moyano *et al.*, 2013).

Whereas microorganisms, which can grow in high concentrations of organic solute, especially sugars, have been called osmophiles, because these organisms do not have an essential demand for reduced (a_w) or high osmotic pressure, rather they tolerate drier environments better than non osmotolerant species (Stecchini and Beuchat, 1985).

Levansucrase (β-2, 6-fructan: D-glucose-1- fructosyl transferase, E.C.2.4.1.10) are involved in the synthesis of fructan polymers known as Levan (Fructooligosaccharides). Levan has potential in the pharmaceutical and food industries (Esawy *et al.*, 2013; Ruhmann *et al.*, 2015). Several applications of levan are as follows: as an agent to increase the viscosity, stabilizer, emulsifier and water-binding to determine and modify the structure of food (Belghith *et al.*, 2012). In pharmaceutical industries, levan is used as a drug carrier agent, anti-hyperglycemia, anti-diabetics (Ahmad *et al.*, 2015), anti-tumor (Dahech *et al.*, 2012), anti-virus (Srikanth *et al.*, 2015) and anti-oxidants (Dahech *et al.*, 2013). In the environmental field, levan can be used as bio-sorbent and bio-flocculants (Sogotcu *etal.*, 2012).

The research goal to study the efficiency of moderately halophilic bacteria isolated from Sawa Lake in the production of levansucrase.

MATERIALS AND METHODS

Isolation of Bacillus isolates:

The sample of Sawa Lake was heat-treated (80ºC for 10min) and individually subcultures on nutrient agar plates, then incubated for 24h at 30ºC and colonies were recovered and purified by streaking on fresh nutrient agar.

Screening of moderately halophilic Bacillus isolates:

Ten concentrations of sodium chloride (1, 3, 5, 7, 10, 12, 14, 16, 18, and 20)% with nutrient agar were prepared and inoculated by Sawa Lake isolates, then incubated for 24h at 30ºC.

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Screening of osmophilic Bacillus isolates:

Nutrient agar with (1, 3, 5, 7, 10, 12, 14, 16, 18, and 20)% of sucrose were prepared and inoculated by Sawa Lake isolates, then incubated for 24h at 30ºC.

Fermentation media:

Fermentation media with the following composition (gm/l) was used for levansucrase production and biomass calculated: K_2HPO_4 (1.5), KH_2PO_4 (1.5), $MgSO_4.7H_2O$ (0.2), NH_4Cl (0.5), yeast extract (2), media were supplemented with 50ml of $CaCl_2$ (5mM) and (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100)gm of NaCl, (Sucrose+Sodium chloride) and sucrose, respectively, then the media were completed with distilled water to one liter, pH was adjusted to 7.0 before autoclaving.

Levansucrase production:

Screening on solid media:

In the present work *Bacillus* isolates were activated in Brain Heart Infusion broth (B.H.I.), 0.1ml of culture was streaked on tryptone sucrose agar medium, composed of (gm/l): sucrose 20, yeast extract 4, tryptone 17, K_2HPO_4 2.5, agar 2, pH 7.0, then plates were incubated at 30°C for 48h.

Levansucrase Activity Assay:

Cells were harvested by refrigerated centrifuge at 5000rpm for 10min after inoculated fermentation media with *Bacillus* isolate and incubation for 48h at 30ºC, then the supernatant was used as enzyme source. Levansucrase activity was assayed by measuring the reducing sugar liberated during sucrose hydrolysis. The reaction mixture [250µl of enzyme extract and 250µl of 1M sucrose in acetate buffer (50mM, pH 5.0)] was incubated at 30ºC for 30min. Reducing sugar released was determined and measured absorbance at 575nm according to the method of (Miller, 1959). One unit of levansucrase activity was expressed as the amount of enzyme required to liberate 1µmol of reducing sugar from sucrose in 1min under experimental conditions.

RESULTS AND DISCUSSION

Collection of samples:

Sawa Lake water (TDS 20800ppm) was heat-treated to kill all vegetative cells, then cultured on nutrient agar to allow germination and growth of heat-resistant spores. Morphological diagnoses of pure isolates were characteristics and determined by microscopy (Gram and spore staining). Four spores-former isolates (Saw 1, Saw 2, Saw 3 and Saw 4) were isolated after incubated for 24h at 30ºC.

The ability of Bacillus isolates growth on various concentrations of NaCl:

Results showed that *Bacillus* isolates (Saw 2 and Saw 3) were responded to various concentrations of NaCl and grown up to 14% of NaCl concentrations with nutrient agar, whereas isolates (Saw 1 and Saw 4) tolerant to NaCl concentrations reaching to 12%, after incubating for 24h at 30ºC (Table 1).

Microorganisms have to equilibrium the vital osmotic gradient across their cytoplasmic membrane indirectly by influencing the osmotic capability of the cytoplasm to coordinate the motion of water in or out of the cell. They accumulate water-attracting ions and organic osmolytes, when they encounter hyperosmotic conditions to prevent cellular dehydration (Bremer and Kramer, 2000) and they quickly remove these compounds through the transient opening of mechanosensitive channels to avert cell rupture (Haswell *et al.*, 2011).

Table 1: Growth of *Bacillus* isolates in various concentrations of NaCl

Conc. of NaCl %	Saw 1	Saw 2	Saw 3	Saw 4
1	R	R	R	R
3	R	R	R	R
5	R	R	R	R
7	R	R	R	R
10	R	R	R	R
12	M	R	M	M
14	S	M	M	S
16	S	S	S	S
18	S	S	S	S
20	S	S	S	S

R: Resistant M: Moderate S: Sensitive

The effect of different concentrations of sucrose on Bacillus isolates growth:

Results indicated that *Bacillus* isolates (Saw 1, Saw 2, Saw 3 and Saw 4) were resisted to different concentrations of sucrose reaching to 200gm/l with nutrient agar after incubating for 24h at 30ºC (Table 2).

Table 2: Effect of various concentrations of sucrose on *Bacillus* isolates

Conc. of sucrose %	Saw 1	Saw 2	Saw 3	Saw 4
1	R	R	R	R
3	R	R	R	R
5	R	R	R	R
7	R	R	R	R
10	R	R	R	R
12	R	R	R	R
14	R	R	R	R
16	R	R	R	R
18	R	R	R	R
20	R	R	R	R

R: Resistant

Many osmophile microorganisms belong to yeast and bacteria were adapted to high osmotic pressure environments, such as high sugar concentrations. Osmophiles are similar to salt-loving microorganisms because a critical aspect of both types of environment is their low water activity (a_w). High sugar concentrations were the growth-limiting factors for many microorganisms, however, osmophiles protect themselves against this high osmotic pressure by the synthesis of osmoprotectants, such as alcohols and amino acids (Beuchat, 1981).

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It is likely that the synthesis and accumulation of glycerol, the poly of choice for osmotic adjustment in xerophilic fungi in high salt (low water-activity) (Lima Alves *et al*., 2015), could facilitate fungal colonization in very salty environment by alleviating against, osmotic stress, low water-activity and ionic strength.

Measurement of cell biomass concentration:

Cell biomass of *Bacillus* isolate (Saw 2) was determined after incubating at 30°C for 48h in media with (NaCl), (NaCl+Sucrose) and (Sucrose), concentrations up to 10%, respectively, and quantified as a milligram of the dry weight of (viable and dead) cells per 1ml of sample. The cells in a sample can be separated from the broth and thoroughly dried before weighing.

Results showed that cell biomass of (Saw 2) were enhanced under high concentrated of (NaCl), (NaCl+Sucrose) and (Sucrose), at (1-10)gm/100ml, respectively, with more affected and increasing in value of cell biomass during add mixture of (NaCl+Sucrose) to fermentation media (Figure 1).

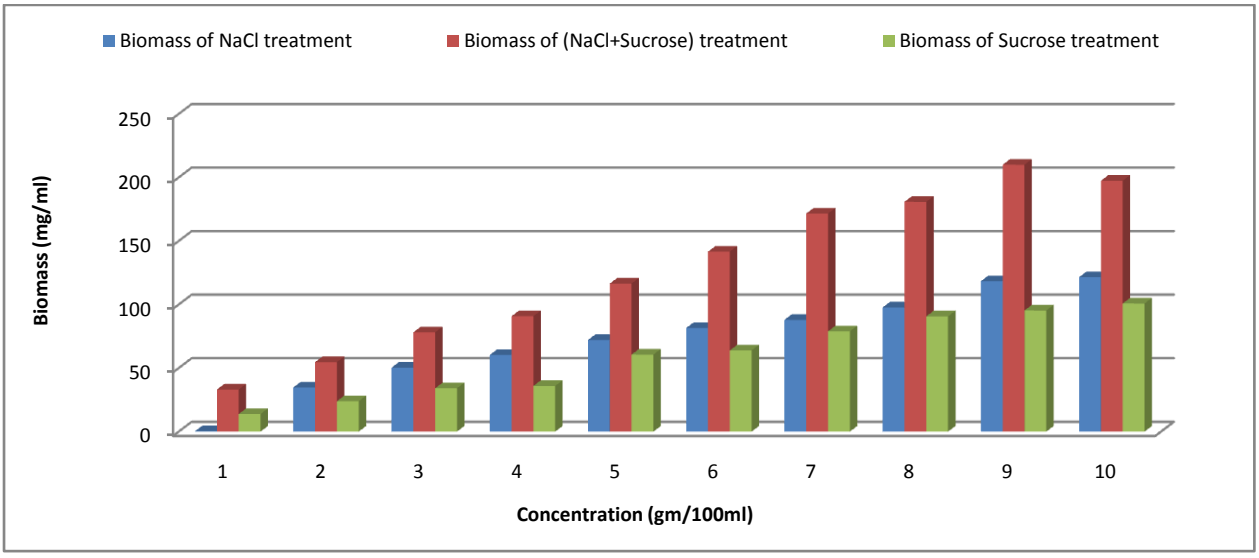

Fig. 1: Effect of (1-10)gm/100ml from NaCl, (NaCl and Sucrose), and Sucrose on cell biomass of (Saw 2) isolate after incubating at 30°C for 48h.

This evidence exhibited that *Bacillus* isolate(Saw 2) can grow within a wide rangeof solute concentrations,ashighosmoticpressure environments. Generally microorganisms capable of growing at water activity (a_w) value 0.85 or less. Well known, mechanisms employed by these organisms to overcome increased osmotic pressure are the intracellular solute accumulation and altered membrane permeability (Pettersson and Leong, 2011).

Qualitative assay for levansucrase:

Appearance of mucous consistency of bacterial colonies on the sucrose agar medium by using sucrose as carbon source after incubating at 30°C for 48h, was the indicator of secreted inducible extracellular levansucrase (Figure 2), which catalyze the exchange of the fructosyl unit of sucrose to various acceptors including sucrose, water and fructan polymer (Perez-Oseguera *et al.*, 1996).

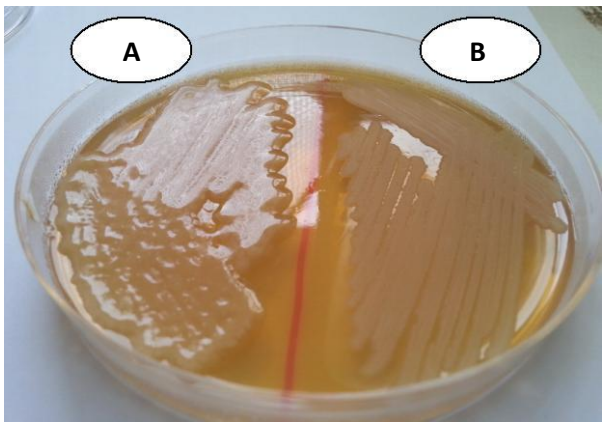

Fig. 2: Exopolysaccharide production (Levansucrase production) A- Positive B- Negative

Quantitative assay for levansucrase:

In the present work, it was trained to analyze the ability of moderately halophilic isolate (Saw 2) to produce levansucrase by utilizing sucrose as a sole carbon source. The levansucrase activity in cell free supernatant was estimated after incubated at 30°C for 48h and showed that (Saw 2) isolate produced levansucrase at concentrations (1-10)gm/100ml of (NaCl+Sucrose), with activity, (462.08, 463.33, 460.83, 460.83, 455.41, 453.75, 452.97, 454.58, 454.16 and 452.07)U/ml and descending quantity in enzyme activity (439.37, 450.41, 431.66, 422.29, 381.04, 361.87, 312.29, 315, 276.25 and 251.87)U/ml when added sucrose concentration to fermentation media more than 4gm/100ml (Figure 3).

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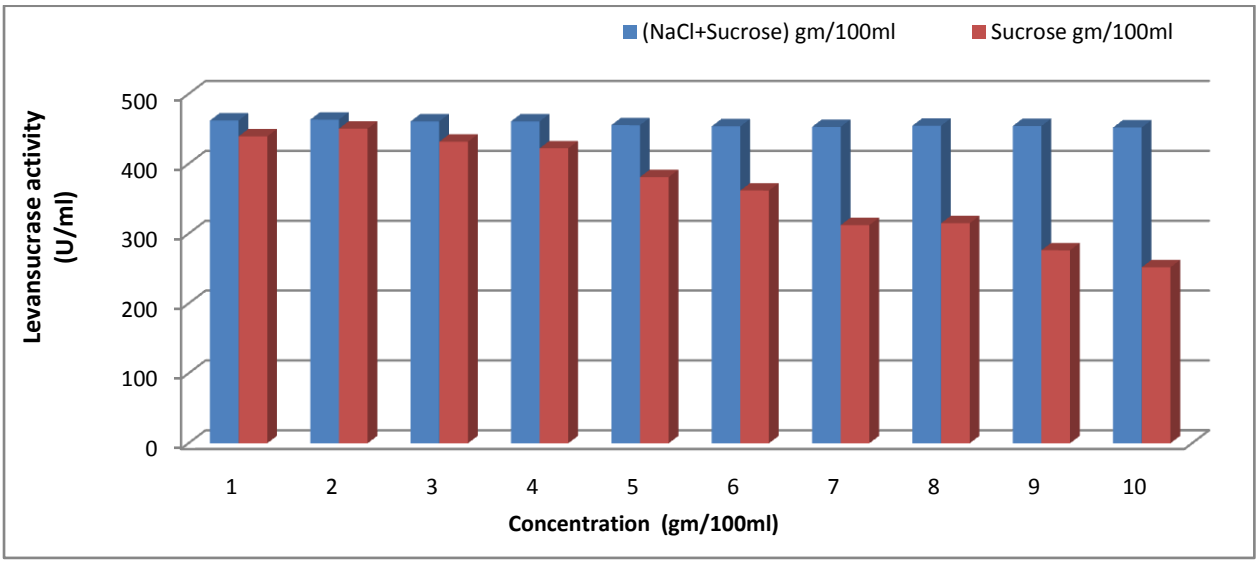

Fig 3: Levansucrase activity (U/ml) producing by (Saw 2) isolate inoculated media with (NaCl+Sucrose) and (Sucrose) (1-10)gm/100ml after incubating at 30°C for 48h.

CONCLUSIONS

This study showed that moderately halophilic *Bacillus* isolates from hypersaline environments able to grow and produced levan via liberated levansucrase under salt and sucrose conditions, reached to 100gm/l, after incubating at 30°C for 48h. The availability of moderately halophilic bacteria was in producing important material, which used in several applications ranging from food and feed (prebiotics, stabilizer, fat substitute), cosmetics (whitener, moisturizer), to pharmaceuticals (anti-oxidant, anti-inflammatory, anti-cancer activities) industries.

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