Increased Fructosyltranseferase (levansucrase) Production by Optimizing Culture Condition from *Pediococcus acidilactici* strain in Shaking batch Cultures

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Abstract: Microbial fructosyltransferases (levansucrases) are polymerases that are involved in microbial fructan (levan, inulin and fructo-oligosaccharide) biosynthesis. Levan has potential importance in food, pharmaceutical, medicine, cosmetics and textile industry. Structurally, microbial fructosyltransferase proteins share the catalytic domain of glycoside hydrolases 68 family and are grouped in seven phylogenetically related clusters. The aim of the work was screening the potency of some bacterial isolates to produce extracellular levansucrase. Bacteria were isolated from different sources (oral swab, spoiled milk, spoiled whey, and spoiled yoghurt). *Pediococcus acidilactici* showed the highest levansucrase activity after 2 days fermentation under shacking condition yielding (12.64 U/ml), and maximum levan production (15.4 g/L). Following the optimization of carbon source, nitrogen source, temperature and initial pH of the growth medium in submerged liquid cultures. In fact, All carbon sources induced the production of levansucrase activity, but sucrose at 30% (w/v) was the most efficient inducer. The optimal temperature and pH of the levansucrase were 30°C and 6.0, respectively. The crude levansucrase of *Pediococcus acidilactici* extract was enriched using selected precipitating agents. The results show that ethanol (65% saturation), provided the highest purification factor of 3- fold with a recovery yield of 41.05% as a total highest recovered protein. The optimum reaction temperature and pH of semi purified levansucrase were 40°C, 5.2 respectively. Analysis of the partial purified enzyme preparation by SDS–PAGE revealed one protein band showing levansucrase activity, The molecular weight of this band was estimated to be around 50.000 Daltons.
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1. Introduction

Levansucrases (β-2, 6-fructan: D-glucose-1-fructosyltransferase, E.C.2.4.1.10) are involved in synthesis of fructan polymers known as levans (fructo-oligosaccharides). Levans are derived from sucrose, consist of linear or branched chains of fructose units attached to sucrose by β (2,6) glycosidic bond (Hernandez and Banguela 2006). Levans are used as viscosifier, stabilizer, emulsifier, gelling or water-binding agent in food, cosmetics and nutraceutical industries (Rairakhwada et al*.,* 2010). Of emerging importance is the use of fructo-oligosaccharides as health-promoting prebiotics, which act as food sources for beneficial bacteria, such as bifidobacteria. The industrial use of levansucrases has long been hampered by costly production processes which rely on mesophilic bacteria (Kucukasik et al., 2011).

Levansucrases catalyze two different reactions - hydrolysis of sucrose when water is used as the acceptor and transglycosylation to form fructose polymers releasing glucose (Ozimek et al*.,* 2006). They are produced by several microorganisms (Bezzate et al., 2000; Morales-Arrieta et al., 2006; Hernalsteens and Maugeri 2008; Van Hijum et al., 2004). Levansucrases have been identified in a number of gram- negative species such as *Erwinia amylovora* (Gross et al., 1990), *Zymomonas mobilis* (Lyness and Doelle 1983), *Acetobacter suboxydans* (Loitsyanskaya et al., 1971), *Gluconobacter oxydans* (Elisashvili 1980) as well as in gram-positive bacteria such as *Bacillus subtilis*, *Bacillus amyloliquefaciens* (Ma¨ntsa¨la¨ and Puntala 1982), *Bacillus natto* (Takahama et al., 1991), *Streptococcus* mutans (Sato et al.,1984). In bacteria, fructan biosynthesis is somewhat simpler because only one multifunctional enzyme is involved.

This enzyme is named levansucrase (EC 2.4.1.10) when the product is a fructan of the type levan (β-2,6-linked polyfructan) or inulosucrase (EC 2.4.1.9) when the fructan is of the type inulin (β-2,1-linked polyfructan). According to Hettwer (1995) levansucrase conducts three characteristic reactions: Synthesis of levan from sucrose by transfructosylation while releasing glucose, hydrolysis of levan to monosaccharides of fructose, exchange of [^{14}C] glucose in the reaction of fructose-2,1-glucose+ [^{14}C] glucose to fructose-2, 1-[^{14}C] gluose+glucose.

Lactic acid bacteria (LAB) comprise an ecologically diverse group of microorganisms united by formation of lactic acid as the primary metabolite of sugar metabolism. The term Lactic Acid Bacteria (LAB) is conventionally reserved for genera in the order *Lactobacillales*, which includes *Lactobacillus,*

Leuconostoc, Pediococcus, Lactococcus and *Streptococcus*. LAB produce a wide variety of anti-microbial factors that include as many metabolic end-products (lactic acid, diacetyl, acetic acid) as bacteriocin peptides (De Vuyst and Vandamme, 1992; Klaenhammer, 1988). Pediocin production by *P. acidilactici* has also been investigated in solid-state fermentation (Vazquez Alvararez et al., 2004). The antimicrobial activity (bacteriocin) against a wide range of Gram-positive bacteria and the minimum inhibition concentrations of pediocin L50, produced by *P. acidilactici* L50 (Cintas et al., 1995). Antibacterial efficacy of bacteriocin was proved against some food spoilage and human pathogenic bacteria like *Enterococcus, Leuconostoc, Listeria, Staphylococcus* and *Streptococcus* (Ferreira et al., 2006). Lactic acid bacteria (LAB) are numerous, and each have a unique role in the amazing conversion of raw materials into lacto-fermented foods. Without LAB, there would be no lacto-fermentation of foods, one of the oldest methods of natural-food preservation (Anthoula et al.,2013). Among the known *Pediococcus* strains, *P. acidilactici*, *P. pentosaceus*, and *P. halophilus* are mostly associated with food fermentations. *P. acidilactici* and *P. pentosaceus*, take place in food fermentations either as indigenous microflora or in starters and both have been used in natural and controlled fermentations of vegetables and sausages (Knorr, 1998).

Microbial levansucrases use different acceptors *in vitro*, such as water (in sucrose hydrolysis reactions), glucose (in interchange reactions) and sucrose or fructan (in polymerization reactions) (Vela´zquez-Herna´ndez et al., 2009). Levansucrases from *Zymomonas mobilis*, *Rahnella aquatilis* JCM-1683, *Pseudomonas syringae* and *Bacillus* sp.TH4-2 are found to be thermostable (Sangiliyandi et al., 1999; Seo et al., 2000; Hettwer et al., 1995; Ammar et al., 2002). However, optimum temperature for levan production was not as high as their hydrolytic activity except for *Bacillus* sp. TH4-2 (Ammar et al., 2002). The industrial use of levansucrases has long been hampered by costly production processes which rely on mesophilic bacteria (Kucukasik et al., 2011). The present study illustrates the characterization of levansucrase *Pediococcus acidilactici* which efficiently converts sucrose to levan.

2. Material and Methods

Isolation of levansucrase producing microbial system

In the present work 6 bacterial isolates (*Bacillus* sp., *Pediococcus* sp., *Streptomyces* sp., *Leuconostoc* sp., *Pseudomonas* sp. and *Streptococcus* sp.) were screened for the levansucrase activity on tryptone sucrose agar medium, it was composed of (g/l) sucrose, 20; yeast extract, 4; tryptone, 17; K_2HPO_4, 2.5; Bacto-agar, 20; the pH was adjusted to 6.0. Those microorganisms were isolated from different sources as oral swab, spoiled milk, spoiled whey, and spoiled yoghurt. The stock cultures of the bacteria were maintained on tryptone medium slants. The inoculated slants were incubated at 37°C for 2 days then stored at 4°C until used. *Pediococcus* sp. was isolated from spoiled yoghurt and found to have maximum levansucrase activity. The bacterial isolate suggested being *Pediococcus acidilactici* according to key of "Bergey's Manual of Systematic Bacteriology, 1986 & Bergey's Manual of Determinative Bacteriology, 1994", and as identified in Al Azhar University, Fermentation, Biotechnology and Applied Microbiology (Ferm-BAM) Center.

Fermentation medium

Unless otherwise specified, the following medium was used for levansucrase and levan production. This medium had the following composition (g/l): Sucrose, 100; K_2HPO_4, 1.5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.2; NH_4Cl, 0.5; yeast extract, 2; completed by distilled water to one liter and the pH was adjusted to 6.0 before autoclaving.

Preparation of the crude enzyme

At the end of the incubation period, the bacterial cells were separated from the culture by centrifugation at 6,000 rpm for 20 min in a cooling centrifuge (Chilspin made in England) at 4°C. The clear supernatant was considered as the crude enzyme source.

Recovery and determination of levan

For levan production, enzyme assay mixture was incubated at 40°C for 12h. Levan was separated by adding 4 volume of chilled 70% (v/v) aqueous ethanol. Mixed by inversion and centrifuged at 6,000 rpm at 4°C for 25 minutes. The pellets washed twice with ethanol and transferred to a weight vial then dried at 60°C to constant weight (MCNeial and Kristiansen, 1990; Thomas and Reed/Hamer,1994).

Estimation of protein content

The protein content of the enzyme preparation was determined by the method of (Lowry et al., 1951).

Levansucrase activity assays

Sucrose conversion by levansucrase yields (a) fructose, which is (partly) built into the growing polymer, and (b) glucose, in a 1:1 ratio to the amount of sucrose converted. In control experiments the glucose formed reflected the total amount of sucrose utilized. Many assays of levansucrase have been based on measurements the amount of glucose released. This was done according to the reaction mixture (1 ml) containing 0.5 ml culture filtrate + 0.5 ml 0.05M acetate buffer in which 0.3 gm sucrose was dissolved at pH 5.2 solution were incubated in water-bath at 40°C for 30 min, 1 ml dinitrosalycilic acid (DNS) was added to the reaction mixture. This mixture was boiled for 15

minutes and then the absorbance was measured at 575 nm. The released glucose due to levansucrase activity was determined by dinitrosalicylic acid (DNS) method (Miller, 1959). One unite of levansucrase activity is defined as the amount of enzyme, which produced 1 µ mole of glucose per ml under the assay conditions.

Immobilization of bacterial cells by gel entrapment

Entrapment in Ca-alginate:

Entrapment of cells within spheres of Ca^{2+} alginate has become the most widely used technique for immobilizing living cells. Bacterial cells were entrapped in 2% calcium alginate gel beads according to the procedure described by (Eikmeier et al., 1984). Two percent sodium alginate solution was prepared by dissolving 2g in 90 ml distilled water and then autoclaving at108°C for 10 min 10 ml bacterial suspension obtained from 2 days old slant culture was added to the sterile alginate solution to obtain 2% final concentration. Ten ml of the alginate bacterial mixture were drawn with the aid of sterile syringe and allowed to drop through the needle into a cross linking solution (100 ml of 2% $CaCl_2$ solution) in 250 ml Erlenmeyer flask to obtain spherical beads (3-4 mm diameter of calcium alginate gel entrapping the bacterial cells). The beads were left in the calcium chloride solution for 1 hour for complete hardening and then washed several times with sterile distilled water the resulted beads from 10 ml alginate were added to 50 ml sterile medium in 250 ml Erlenmeyer flask. The flasks were shacked at 30°C.

Entrapment in agar:

The wet cell pellets obtained from 100 ml culture of *Pediococcus acidilactici* were mixed with 10 ml of 2, 3 and 4% (w/v) agar solution at 45°C. The mixture was quickly cooled to 4°C, cut into 2 × 2 × 2 cm^3 fragments (Cheetham et al., 1985). The cubes obtained from 10 ml gel were used for inoculation of 100 ml of the medium. At the end of the fermentation period, the culture was decanted and the supernatant was investigated for levansucrase activity.

Partial purification of levansucrase enzyme produced by *Pediococcus acidilactici*

Partial purification was achieved by salting-out with ammonium sulphate and by fractional precipitation with acetone and ethanol. The crude culture supernatant obtained from 2 days old cultures of *Pediococcus acidilactici,* grown under optimal conditions (sucrose 300g, KH_2PO_4 2g, K_2HPO_4 1.25g, KCl 0.5g, $(NH_4)_2SO_4$ 0.4g, Mg SO_4 0.18g). The medium was adjusted to pH 6.0 and 30°C was precipitated at different concentrations of each precipitant in a sequential manner.

Salting-out with ammonium sulphate:

250 ml volume of the crude culture supernatant was fast rotated in a cooling centrifuge 6,000 rpm at 4°C to remove the residual waste and bacterial cells. The protein content and the levansucrase activity of the enzyme solution were determined as described previously. The whole enzyme solution was kept in an ice bath. This was followed by adding ammonium sulphate very slowly while stirring to the ice cold enzyme solution until the desired saturation of ammonium sulphate was reached. The solution was left for 2 h and then centrifuged for 15 min at 7,000 rpm in a cooling centrifuge. The precipitate (Fraction 1) was removed and further ammonium sulphate was added to the supernatant fluid to obtain the next fraction. The process was repeated until 100% saturation was reached. Each precipitated fraction was dissolved in about 10 ml distilled water and dialyzed against distilled water (or buffer) in a cellophane bag in a refrigerator until the water outside the bag gives no precipitate with 1% barium chloride solution, indicating that the enzyme solution inside the bag become free of sulphate. This was achieved by changing water (or buffer) outside the bag several times. After complete dialysis, each enzyme solution was dried. The protein content and levansucrase activity of each solution was measured.

Fractional precipitation with acetone

Acetone (Analar Reagent) was cooled at 4°C one day before starting the precipitation. The whole enzyme solution was kept in ice bath; a certain volume of acetone was added slowly while stirring until the required concentration was reached. After removing the precipitated fraction by centrifugation at 5,000 rpm at 4°C for 15 min in cooling centrifuge, further acetone was added to the supernatant fluid and the process was repeated until the acetone reached a final concentration of 85%, several enzyme fractions were thus obtained (25%, 35%, 50%, 65%, 75%, and 85%). Acetone fractions were dried over anhydrous calcium chloride under reduced pressure at room temperature and then dialyzed against distilled water. Each enzyme fraction was assayed for levansucrase activity and protein content.

Fractional precipitation with ethanol

Absolute ethanol (Analar Reagent) was cooled at 4°C over night and precipitation with ethanol was performed using the same procedures as that of acetone precipitation.

Sodiom dodecyl sulfate - polyaccylamide gel electrophoresis (SDS-PAGE)

For the determination of homogeneity and molecular weight, the enzyme preparations and protein markers were subjected to electrophoresis by the method of Bollag and Edelstein (1991) with the use of 10% acrylamide gel. Sucrose (0.2%) was incorporated into the separating gel prior to the addition of ammonium persulphate and polymerization. After electrophoresis, the gel was stained with Coomassie Blue R dye in methanol- acetic acid–water solution

(4:15, by volume) for 1h and distained in the same solution without dye. For the activity staining of levansucrase activity, SDS was removed by washing the gel at room temperature in solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%) for 1h and solution B (sodium phosphate buffer, pH 7.2) for 1h respectively. Renaturation of the enzyme proteins was carried out by leaving the gel in solution C (sodium phosphate buffer, pH 7.2, containing 5mM b- mercaptoethanol and 1mM EDTA) at 4°C overnight. The gel was then transferred on to a glass plate, sealed in a film, and incubated at 37°C for 5 h. The gel was stained in a solution of 1% Congo Red for 30 min, and distained in 1 M NaCl for 15 min. Clear bands indicated the presence of levansucrase activity. Each sample was applied to a separate well in the slab gel along with a pertained SDS molecular weight marker (14-205 K Daltons).

3. Results

Survey of the bacterial isolates for the production of extracellular levansucrase

In the present work it was aimed to study the ability of some locally isolated bacterial strains to utilize sucrose as a sole carbon source to produce levansucrase throughout production of levan. The culture medium was distributed in 250 ml Erlenmeyer flasks (50 ml/ flask) and sterilized (120°C, 20 min). After sterilization, the flasks were inoculated with 1 ml bacterial suspension obtained from a 2 day old slant culture. Incubation was carried out at 30°C in an orbital shaker (160 rpm) for 2 days. The levansucrase activity present in the culture filtrates was estimated as previously described. The results showed at Fig.1, that all the tested bacterial strains produced a levansucrae activity, the most highest value was that obtained in cultures of *Pediococcus acidilactici* while the lowest was that produced by *Streptococcus* species. The highest polymer production was obtained in cultures of *Pediococcus* sp. and *Leuconostoc* sp. lower production was in *Bacillus* sp. and *Pseudomonas* sp. while production of the polymer was not observed in *Streptococcus* sp. and *Streptomyces* sp. tested cultures.

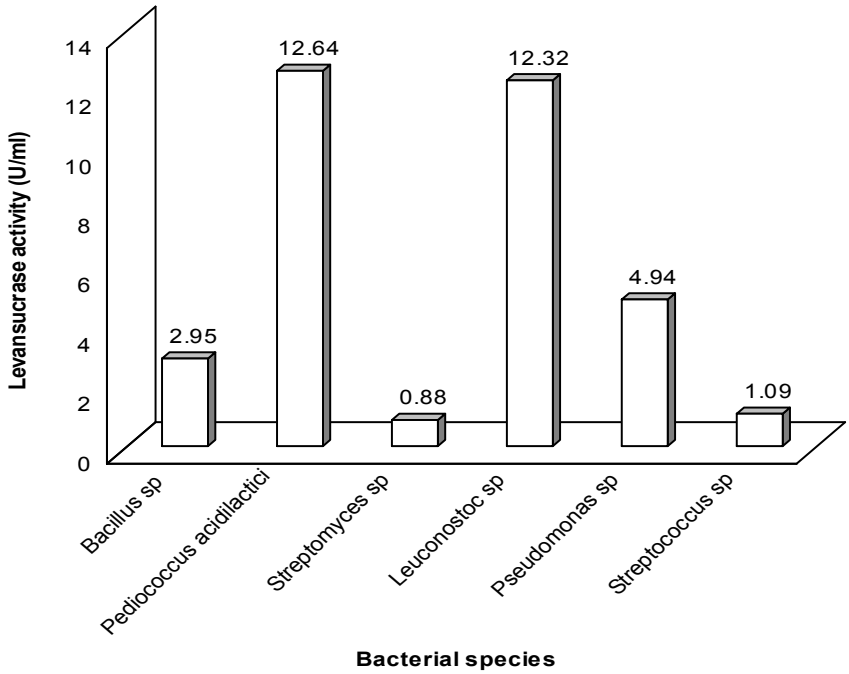

Fig. 1: Production of levansucrase by different bacterial species

Effect of carbon source and different concentrations of sucrose

The effect of carbon source on the production of levansucrase was studied using the basal culture medium supplemented with 10 % sucrose as a control. On other experiments sucrose was replaced by equal carbon amounts of different carbon sources which include glucose, galactose, fructose, maltose, and lactose as a sole carbon source or sugar cane molasses as natural substrate containing the carbon source. The data illustrated in Fig. 2, show that the activity of the enzyme was differently affected by mono and disaccharides sugars as carbon sources. Sucrose was the most preferable carbon source yielding a maximal activity (12.64 U/ml), followed by galactose (11.04 U/ml), on the other hand the lowest activity was obtained in cultures containing lactose (4.91 U/ml). Different concentrations of sucrose (as the carbon

source) ranging from 2.5 to 25 g/flask were individually supplemented to the basal medium. The optimum sucrose concentration was 15 g/flask; yielding a high levansucrase activity (22.69 U/ml) and the highest polysaccharide value 17.65 g/l (Fig. 3).

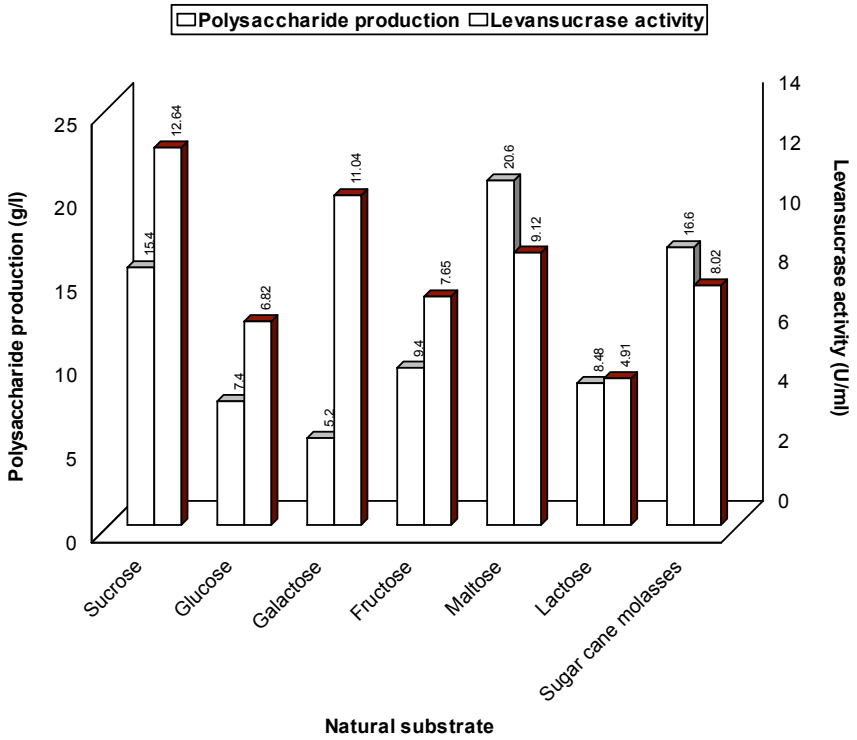

Fig.2: Effect of some carbon sources on the production of levansucrase in *Pediococcus acidilactici* cultures

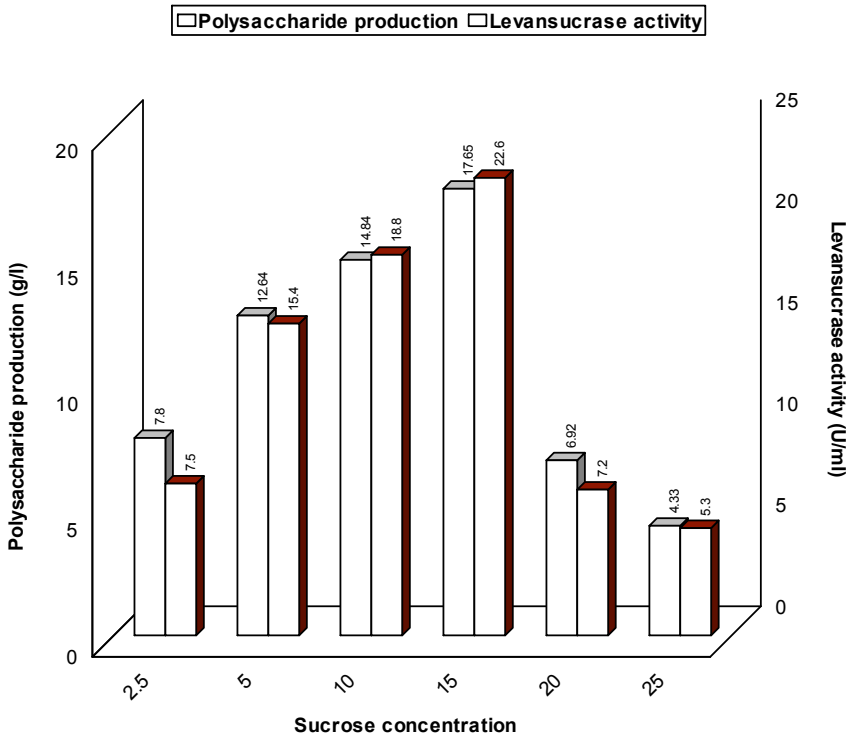

Fig. 3: Effect of different concentrations of sucrose on the production of levansucrase by *Pediococcus acidilactici*

Effect of nitrogen source and ammonium sulphate concentration

Ammonium sulphate 0.1% used as a nitrogen source in the basal medium as described before, was replaced on equal nitrogen basis, by ammonium sulphate, sodium nitrate, urea, peptone, tryptone, yeast extract each at a time. Ammonium sulphate gave levansucrase activity higher than that obtained in all the other tested nitrogen source cultures (Table 1), so it was the most preferable nitrogen source yielding a maximal levansucrase activity of (22.69 U/ml) and polysaccharide production. Different ammonium sulphate concentrations ranging from 0.5 to 2 g/l were individually supplemented to the basal medium containing 30% sucrose (as the sole carbon source). A parallel increase of polysaccharide production and enzyme activity in-agreement with the raising of $(NH_4)_2SO_4$ concentration until 1g/l (Fig.4). The highest levansucrase activity (39.5 U/ml) as well as polysaccharide production (28.49 g/l) were detected at 1g/l $(NH_4)_2SO_4$ concentration.

Table (1): Effect of different nitrogen sources on the production of levansucrase by *Pediococcus acidilactici*

Nitrogen Source	Polysaccharide production (g/l)	Levansucrase activity (U/ml)	Protein content (mg/ml)
NH_4Cl	22.6	17.65	0.82
$(NH_4)_2SO_4$	17.65	22.69	0.69
$NaNO_3$	15.6	12.29	0.66
Urea	10.6	8.52	0.79
Peptone	17.2	16.34	1.33
Tryptone	11.3	9.645	1.07
Yeast Extract	11.6	10.93	1.04

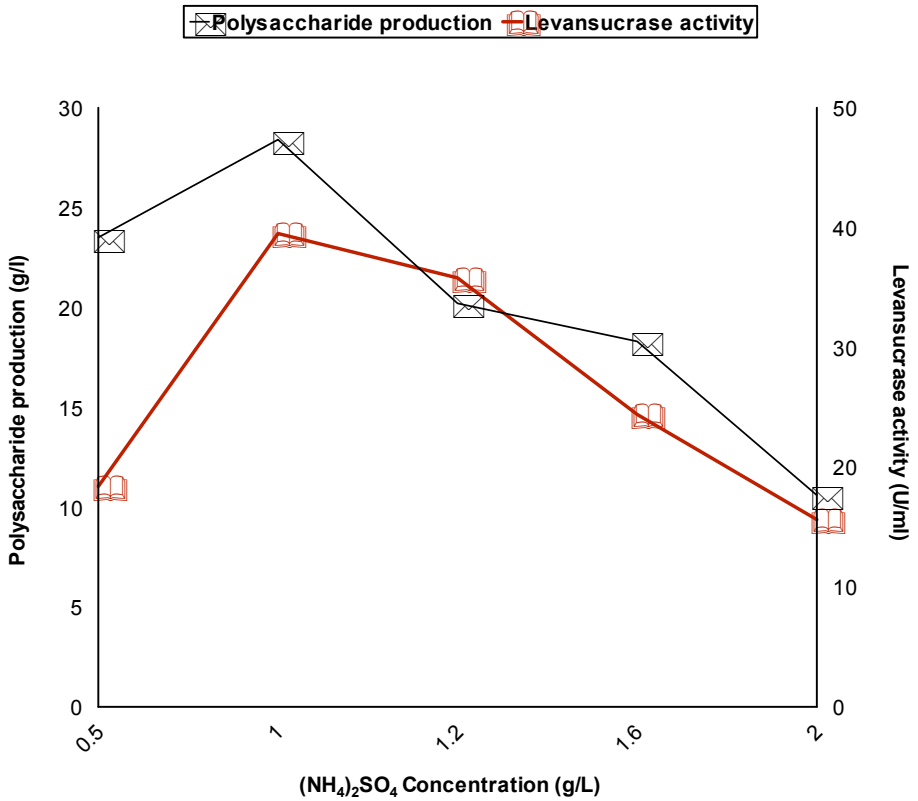

Fig. 4: Effect of different concentrations of $(NH_4)_2SO_4$ on the production of levansucrase by *Pediococcus acidilactici*

Effect of initial pH value of the medium

The medium was initially adjusted to cover a pH range from 4.0 to 9.0. All adjustments were carried out before sterilization of the medium by means of pH-meter. The initial pH 6.0 gave the highest levansucrase activity (39.5 U/ml) and maximum polysaccharide production (28.2 g/l), above and below this value showed an adverse effect on the enzyme activity. Enzyme activity was stable in the pH range 6.0 to 8.0 (Fig.5).

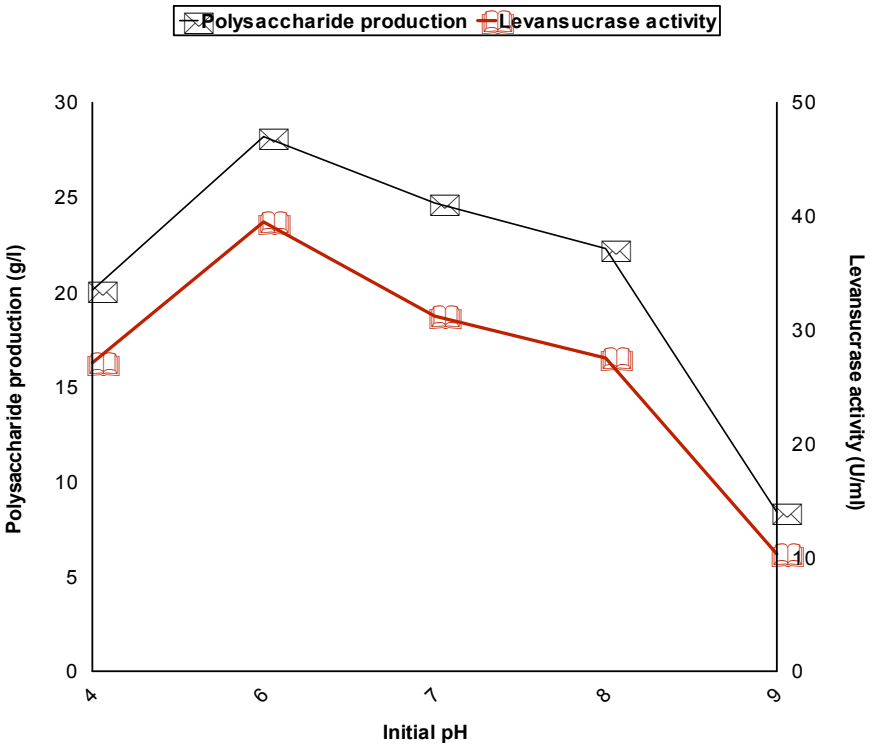

Fig. 5: Effect of initial pH value on the production of levansucrase by *Pediococcus acidilactici*

Effect of inoculum size and incubation period

In this experiment, the fermentation flasks (each containing 50 ml basal medium) received different inocula ranging from 0.5 to 2.5 ml of bacterial suspension obtained from a 2-days old culture of *Pediococcus acidilactici*. The flasks were incubated at 30°C in shacking incubator for 2 days. The highest levansucrase activity (39.5 U/ml) and polysaccharide production (28.2 g/l) were obtained at 1ml bacterial suspension/flask. To investigate the maximum production of extracellular levansucrase during different incubation periods (1,2 and 3). The activity increased regularly during the first 24 hours of incubation and the maximum levansucrase activity (39.5 U/ml) was obtained after 2 days of incubation reading 1.81-fold of that 1 day. At longer incubation periods the activity decreased gradually reaching to (20.38 U/ml) after 3 days.

Effect of some additives

The effect of some additives on the production of levansucrase was studied using the modified culture medium supplemented with sucrose (15 g/flask) (30%) as a carbon source, (NH_4)$_2SO_4$ (1.0 g/l medium) (0.1%), K_2HPO_4 (1.5 g/l medium) (0.15%), KH_2PO_4 (1.5g/l medium) (0.15%), $MgSO_4.7H_2O$ (0.2 g/l medium) (0.02%), KCl (0.5 g/l) (0.05%), pH adjusted at 6.0, inoculum size is 1.0 ml suspension of *Pediococcus acidilactici* incubated for 2 days at 160 rpm shaker. Different additives casein, whey, wheat bran, soy bean meal, were added each at a time, a controlled flask was tested. The data in Table 2 indicate that, the control flask which has no additives showed a higher levansucrase activity than that obtained with all the other tested cultures, the culture containing no additives was the most preferable one yielding a maximal levansucrase activity of (39.5 U/ml) and polysaccharide production (28.2 g/l).

Table (2): Effect of different additives on the production of levansucrase in *Pediococcus acidilactici* cultures

Additives	Polysaccharide production (g/l)	Levansucrase activity (U/ml)	Protein Content (mg/ml)
Casein	8.6	12.82	1.52
Whey	15.1	24.04	2.16
Wheat bran	19.2	30.53	2.49
Soy bean meal	22.4	34.01	1.06
No additives	28.2	39.5	0.61

Effect of entrapping cells in different gel material on the production of extracellular levansucrase from immobilized *Pediococcus acidilactici* cells and the effect of different agar concentrations

The cells were entrapped in alginate or agar and free cells were used as control. An optimized culture medium in 250 ml Erlenmeyer flasks was inoculated with porous beads of the tested gel material including bacterial cells. After 2 days in shaken culture at 30°C, the levansucrase activity, protein content and, levan amount produced were determined for the entrapped and free cells. The results graphically illustrated in Fig. 6, indicated that agar gel cultures recorded the highest levansucrase activity (47.42U/ml) and polysaccharide production (34.6g/l) for the entrapped cells, while alginate exhibited a high protein content (1.1 mg/ml) with low activity than agar and higher than free cells (41.36 U/ml). Effect of different concentrations of agar on the levansucrase production using a concentration range from 2% to 5%..The results illustrated in Fig.7, show that, the highest levansucrase activity (47.42U/ml) was obtained at level 2% with 3.2-fold of that level 5% of agar concentration.

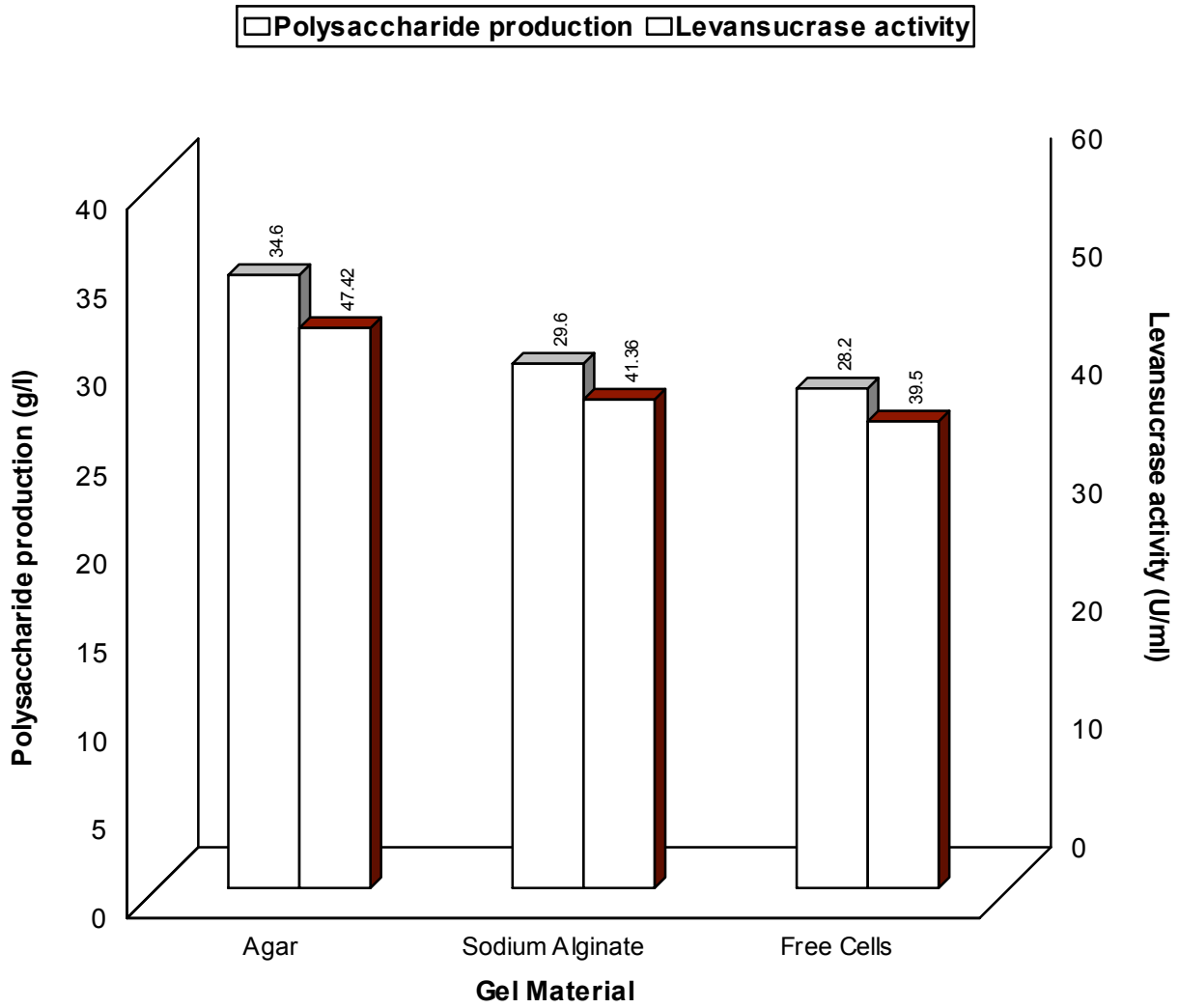

Fig. 6: Production of levansucrase by *Pediococcus acidilactici* entrapped in different gel materials

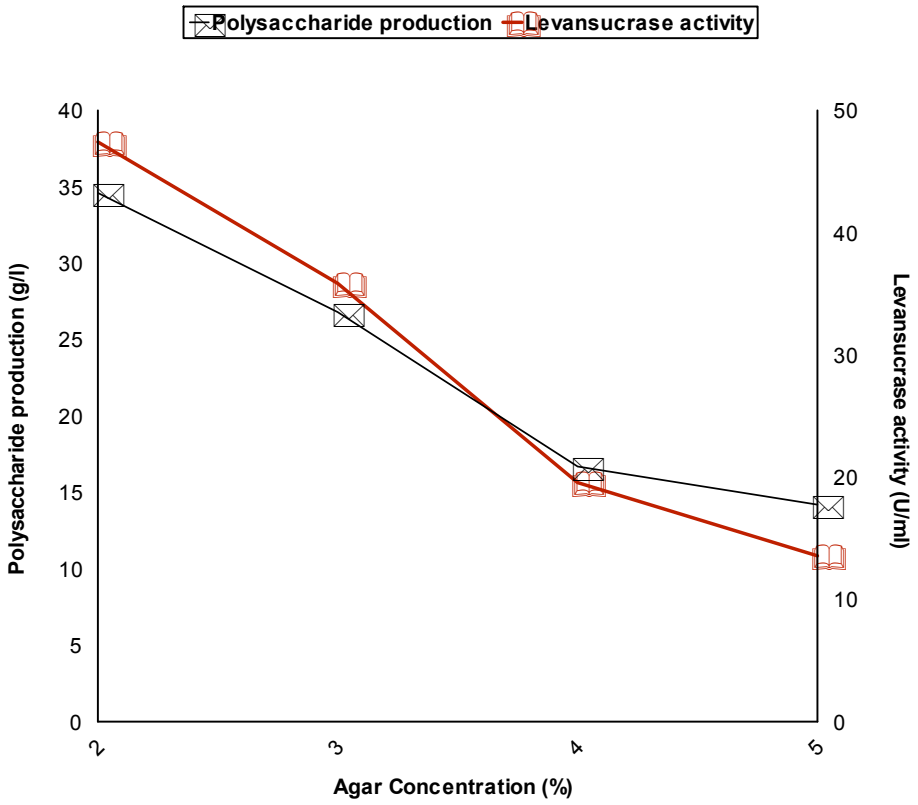

Fig. 7: Effect of different agar concentrations on the production of levansucrase by *Pediococcus acidilactici*

Effect of different inoculum size of agar 2% concentration with entrapped *Pediococcus acidilactici* cells on the production of levansucrase

Effect of different inoculum size of agar 2% concentration with entrapped *Pediococcus acidilactici* cells on the production of levansucrase. The experiment was carried under shacking condition and at 30°C. The result given in Table 3, show that, the best inoculum size for producing a high levansucrase activity (47.42U/ml) was obtained at 25 ml of agar and polysaccharide production (34.6g/l).

Table (3): Effect of different inoculum size of agar on the production of levansucrase by *Pediococcus acidilactici*

Agar Amount (ml)	Polysaccharide production (g/l)	Levansucrase activity (U/ml)	Protein Content (mg/ml)
12.5	17.8	28.62	0.66
25	34.6	47.42	0.65
50	20.5	30.70	0.70
75	16.6	27.91	0.8
Free Cells	28.2	39.5	0.61

Scanning electron micrograph

According to scanning electron microscope with a low magnification power. The gel-entrapped cells grew well inside the gel matrices. The micrographs indicated to the good adsorption of bacterial cells on the surface of supporting material with different degrees. So that, it could be emphasis the explanation of high activity of levansucrase that obtained from entrapping in agar particles than other supporting materials (Fig.8). Some details of morphological characteristic of *Pediococcus acidilactici* immobilized in alginate beads, such as cell shape: cocci, cell arrangement: tetrads, some in pairs with a higher magnification power (Fig.9).

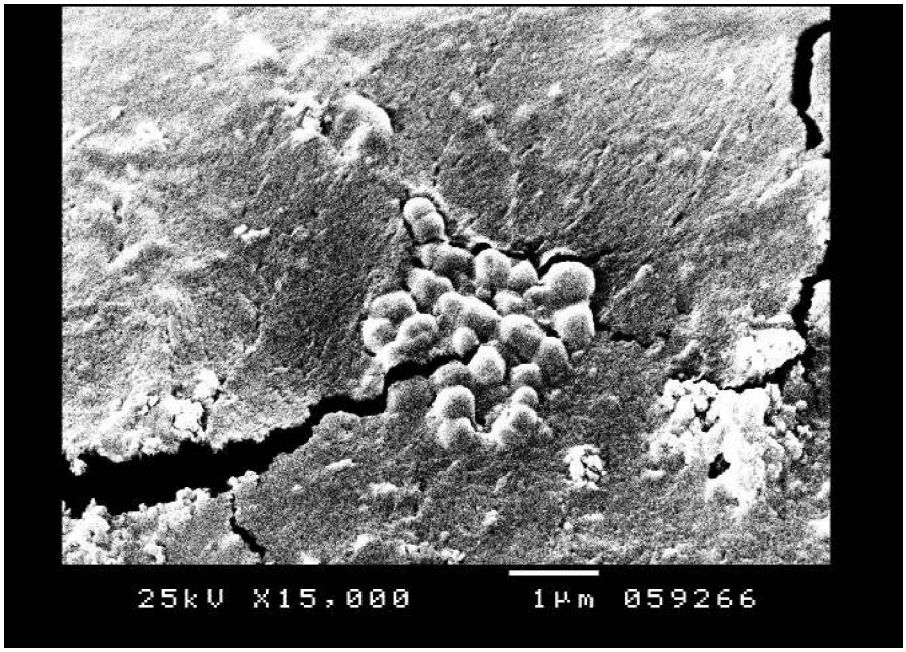

Fig. 8: *Pediococcus acidilactici* cells immobilized on agar.

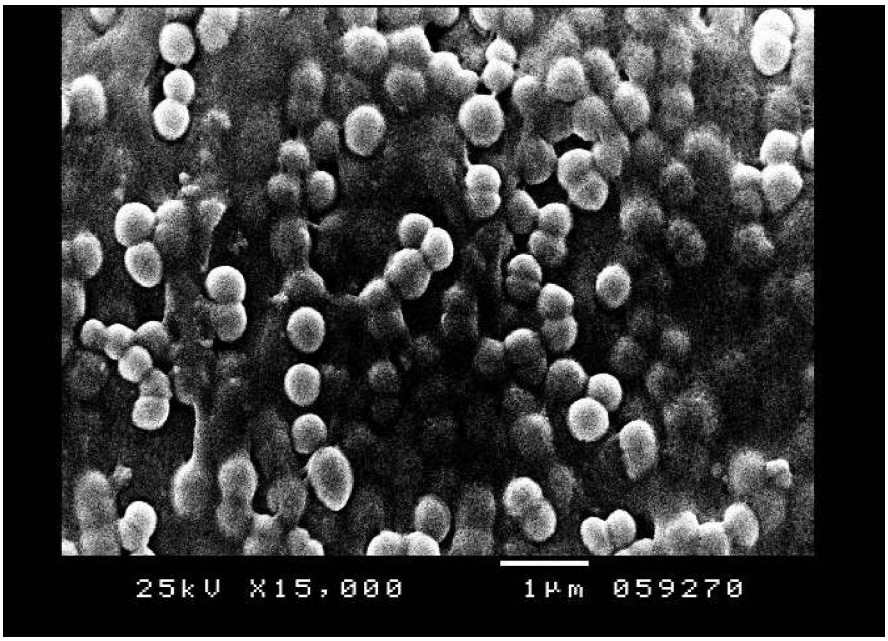

Fig. 9: *Pediococcus acidilactici* immobilized in alginate beads with high magnification power.

Partial Purification of extracellular levansucrase produced *by Pediococcus acidilactici ;* Fractional precipitation by using different precipitation agents

The culture filtrate was centrifuged at 7,000 rpm at 4°C and the clear supernatants were used for fractional precipitation. Several protein fractions were obtained by adding different concentrations of ammonium sulphate, ethanol or acetone, separately to a certain volume of the clear crude enzyme solution. After precipitation with any of the used agents, each fraction was dissolved in water and dialyzed against distilled water in a refrigerator. The protein content as well as levansucrase activity and specific activity of each fraction were determined. The results indicated that fractional precipitation of levansucrase from *Pediococcus acidilactici* cultures by using different precipitation agents comparing to crude enzyme, a total of 11 fractions were obtained where the highest levansucrase specific activity present in the fractions precipitated with ethanol yielding 186.2 U/mg (Fig.10),

followed by ammonium sulphate (159.34 U/mg) and acetone yielding (152.45 U/mg). Most of the obtained fractions showed a specific activity higher than that of the crude enzyme, indicating the presence of purification among all the obtained fractions, the 65% ethanol fraction showed the highest protein content and highest levansucrase activity, as it give about 3-fold of the crude enzyme.

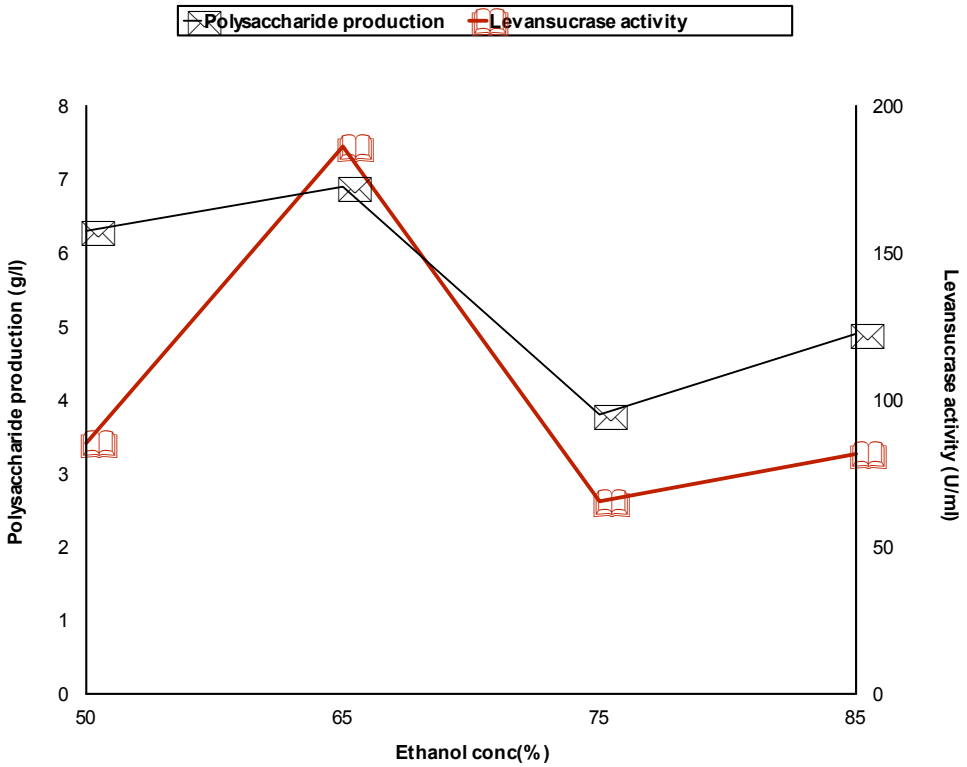

Fig.10: Fractional precipitation of levansucrase from *Pediococcus acidilactici* cultures by using Ethanol

Some properties of the partially purified extracellular levansucrase

Effect of enzyme protein and substrate concentration

The semi pure enzyme tested in a reaction mixture containing 30 mg of sucrose in 0.05 acetate buffer pH 6.0 and different concentration of enzyme ranging from 50 to 800 µg protein/ml reaction mixtures. Every enzyme solution was incubated for 20 min at 40°C. Controls were made at each experimented concentration using heat denaturated enzyme. However, maximum specific activity was obtained at an enzyme protein concentration of 50 µg/ml reaction mixture. Higher protein concentration showed a decrease in the specific activity. The sucrose concentration used in the reaction mixture was varied in a range of 0.1 to 0.6 g/ml reaction mixture. The optimum enzyme concentration was 50 µg enzyme protein and the reaction was carried at 40°C, pH 6.0 for 20 min. The optimum substrate concentration for the semi pure enzyme was 0.2 g/reaction mixture. At this concentration, the highest specific activity was recorded.

Effect of temperature and pH of the reaction

The enzymatic reaction was carried out for 20 min at temperature 30, 40, 50, 60 and 70 °C using an enzyme protein and substrate concentration of 50 µg and 0.2 gm respectively per ml reaction mixture. In each case, a control was made using previously heated enzyme solution in the reaction. The data were indicated that levansucrase activity increased gradually by increasing the reaction temperature reaching its maximum value at 40°C. A pH range of 3.6 to 5.6 was used (acetate buffer, 0.05M). All the other assay conditions were at the optimum value as determined from the previous experiments. The optimum pH value for the semi pure enzyme was 5.2 where the highest specific activity was obtained (293.41 U/mg). Higher or lower pH values showed an adverse effect on the activity and the lowest activity was obtained at pH 3.6 showing about 54.53% decrease of the value obtained at pH 5.2.

Determination of apparent molecular weight

Molecular weight of the partial purified enzyme was determined by SDS- PAGE (12%) as described previously in the materials and methods. Analysis of the enzyme by SDS- PAGE revealed the appearance of

one band with molecular weight of 50.000 Daltons (calculated from the relation between the maker and the relative mobility of the calculated molecular weight), Figure (11).

Rm=

Distance traveled by protein

Distance traveled by font dye

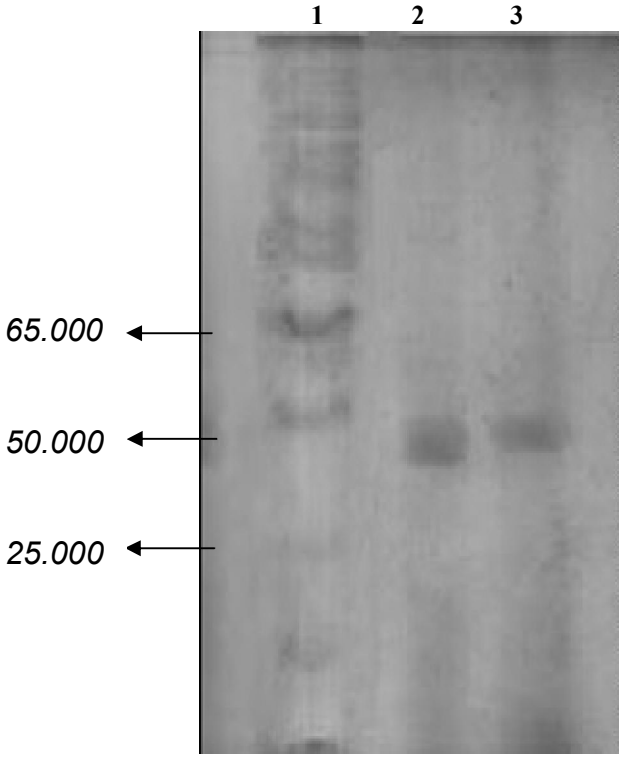

Figure 11: SDS gel electrophoresis of partially purified levansucrase from *Pediococcus acidilactici* (lane 2) and of the crude enzyme (lane 3). Lane one represents molecular weight marker protein. KDa: Molecular weight of marker proteins used (kilo Dalton)

4. Discussions

Levansucrases belong to glycoside hydrolases 68 (GH68) family catalyzing hydrolysis of sucrose and also transfer fructose residues to the suitable acceptors. Bacterial levansucrases are generally known as extracellular proteins, and produce high molecular weight fructans (Seo et al., 2000). In plants, fructans are used as reserve carbohydrates stored in cell vacuoles and their production is induced under a biotic stress viz. high light, cold and drought (Hernandez and Banguela 2006). Levansucrase (EC 2.4.1.10) of various bacteria (Doelle et al., 1993) catalyze the synthesis of levan, the 2, 6-linked fructose polymer. This polysaccharide possess good prospects for several biomedical applications and the Gram-negative ethanologenic bacterium *Zymomonas mobilis* exhibit strong potential for its biosynthesis in sucrose-containing media (Falcao et al., 1993). However, the utilizable substrate range for *Z. mobilis* is restricted to glucose, fructose, and sucrose, and only sucrose should be recognized as an appropriate substrate for levan synthesis. It has been shown that multiform bacterial levansucrase perform the polymerization of fructose moiety from catabolised raffinose (Hetwer, et al., 1995).

Pediococcus strains from cocci in clusters, are negative for gas production from glucose, and are of lactic acid bacteria, Members of these genera have been implicated in bloodstream and other types of infections in compromised hosts (Kathryn, 2002).The highest activity of *Leuconostoc* sp. was (12.32 U/ml) but we need sucrose as a carbon source because the growth in sucrose is followed by extra-cellular formation of fructo-oligomers and levan catalyzed by the levansucrase enzyme that hydrolyzes the sucrose and polymerizes the fructose in levan, (Borsari et al., 2006). The utilizable substrate range for *Zymomonas mobilis* is restricted to glucose, fructose, and sucrose, and only sucrose should be recognized as an appropriate substrate for levan synthesis. It has been shown that multiform bacterial levansucrases can or cannot perform the polymerization of fructose moiety from catabolised raffinose, similar to the concentration of sucrose; the levansucrase catalyzed reaction displayed a sigmoidal response of the reaction rate to the concentration of raffinose with the cell- free extract as an enzyme source (Andersone et al., 2004). Levansucrase hydrolyzes sucrose to produce free glucose and levan; some free fructose is also formed. Two disaccharides lactose and cellobiose inhibit the enzyme (Pabst**,** 1977), other investigators produced levansucrase in the presence of various carbon sources sucrose, glycerol, mannitol (Hernandez et al., 1995), and others used (glucose, sucrose, fructose) as a carbon source on at a time growth occurred on sucrose and glucose but not on fructose and polymer (levan) only on sucrose (Bodie et al., 1985). Sucrose was found as the best substrate for levansucrase production beside it is a specific substrate for production of levan. Which record a high activity (17.65 U/ml) and high levan production (22.6 U/mg), many investigators used sucrose as a sole carbon source for the cultivation of some bacteria producing levansucrase (Ahmed, 2008), as substrate compared to sugar beet molasses (12.4 g l-1) at 30 g l-1 sucrose concentration (Kucukasik et al., 2011). (Fleites et al., 2005) study is to assess sugar cane juice and sucrose as substrates (Borsari et al., 2006). (Viniti and Theertha, 2012) indicated that cane molasses as substrate gave higher yield of levan compared to cane juice. The analysis of the results obtained shows that with the increase in the total sugar concentration from (200 g/L) to (300 g/L) the levan production was decreased to 46.21%, the sugar cane

juice and sucrose interaction had a negative effects inhibiting levan formation. (Vigants et al.,1996) who studied culture medium osmotic pressure ascertained that the levan synthesis decreased significantly when the substrate concentration was increased. (Muro et al., 2000) reported that there were no differences in the levan formation when the sucrose concentration was increased from 200 to 300 g/L.

(Doelle et al.,1993) described that high sugar or salts concentrations caused differences in the use of fructose causing the formation of fructo-oligomers. Aerobic spore forming *Bacillus* were the most frequently encountered microbes on the external surface, cropland intestine of the honey bees and consequently honey (Root, 1993, Esawy et al., 2011) who studied the production of levansucrase from novel honey *Bacillus subtilis* isolates. It can be noticed that the pH 6.0 the most favorable for the Levansucrase activity yielding (39.5 U/ml). In general, some investigators differ as used initial pH 7.0 (Ahmed, 2008, and Bodie et al., 1985), pH 5.8 (Hernandez et al., 1995). It was also noticed that the most ideal incubation period is for 2-days for levansucrase activity yielding (39.5U/ml). Some investigators incubate for 22 hours (Andersone et al., 2004), other incubated for 72 hours at static condition as (Ahmed, 2008).

The biosynthesis of levansucrase by *Pediococcus acidilactici* immobilized by entrapment in different gel materials was briefly studied. It was found that the best concentration was 2% of agar gel material. At this concentration levansucrase activity reached about (47.42 U/ml) which was a high yield in comparison with other gel material. It was reported by (Jang et al, 2001) that the immobilised levansucrase of *Zymomonas mobilis* expressed in *Escherichia coli* retained 61% of the original activity after five repeated uses. Partial purification of the crude levansucrase produced by *Pediococcus acidilactici was* fulfilled by fractional purification with ethanol, acetone and ammonium sulphate. The 65 % ethanol fraction showed the highest protein content and also showed the highest levansucrase activity of ethanol fractions as it give about 3-fold of the crude enzyme which indicate to the highest specific activity. Other investigators used ammonium sulphate for fractional precipitation of Levansucrase from *Actinomyces viscosus* ATCC 15987(Miller and Somers, 1978). The estimated molecular weight of *Pediococcus acidilactici* fructosyltransferases (FTF) falls within the same range as those reported for the FTF (levansucrase) monomers from *B. subtilis* (50 kDa) and *B. amyloliquefaciens* (52 kDa) (Mäntsälä and Puntala, 1982). In contrast, the molecular weight of *Z. mobilis* FTF (levansucrase) was shown to be 94 kDa (Goldman et al., 2008) by size exclusion, while the SDS-PAGE revealed a single protein band of 55 to 56 kDa (Sangiliyandi et al., 1998; 1999). Taking into consideration the molecular weight determined by gel filtration, this suggests that the FTF from *Z. mobilis* exists as a dimer with two subunits of 56 kDa. In fact, the FTF from *R. aqualitis* (Ohtsuka et al, 1992), and *Z. mobilis* (Goldmann et al., 2008) have also been shown to exist in the dimer form.

Conclusion

The results of the present investigation collectively indicate the possibility of using free or immobilized cells of *Pediococcus acidilactici* for high transfructosylation activity, and analyzed its efficiency for levan production using sucrose as a carbon source. Levansucrase from *Pediococcus acidilactici* isolated from spoiled yoghurt was found to be most active at 30°C and pH 6.0, with optimum levanesucrase activity and tolerant for wide range of pH. Levansucrase has a technological importance in food, feed, as well as in medicine (Kang et al.,2009). Results of this study suggest that the levansucrase from *Pediococcus acidilactici* is quite efficient in converting sucrose to levan, which promising as a food additive and in medicine as a plasma substitute, drug activity prolongator and an antihyperlipidemic agent also has anti-diabetic effects (Dahech et al., 2011). It presents low viscosity, high solubility in water, biocompatibility as well as other properties that can have industrial applications such as a hypocholesterolemic agent, an immune modulator agent, an antitumor activity, an anti-inflammatory activity and a blood plasma substitute and extender.

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