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Production of a Novel Halophilic Dextranase from a Honey Isolate, *Bacillus subtilis* NRC-B233 under Solid-state Fermentation

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A GRAM-POSITIVE, sporulating halophilic bacteria, designated ..... NRC-B233, was isolated from the honey produced in Saudi Arabia. It was identified by the 16-23S intergenic region as *Bacillus subtilis* NRC-B233. Screening of the wastes and agro-products for dextranase production under solid state fermentation showed that corn flour was the best substrate (61.323 U/g). The optimum conditions for dextranase productions were 37°C, pH 9, 32 hr incubation period, and 200 % moisture content. The most favorable nitrogen and carbon sources for enzyme production were 2% peptone and 5% starch (1076.768, 1553.364 U/g). respectively. A unique character of this isolate is its ability to continuously produce dextranase in the absence and presence of NaCl 5-20 g/l. The addition of 0.175 Mm $CrCl_3$ increased the dextranase production about 4.5 fold. The enzyme has been partially purified about 112-fold from crude extract by only two purification steps involving ultra-filtration. The purified dextranase showed its maximum activity at pH 9.2 and 70ºC. It retained full activity (100%) at 75ºC for one hour. Dextranase activity increased about four fold in the presence of 10% NaCl. On the other hand, $CaCl_2$ (0.050M), EDTA (0.100M), and KCl (0.100M) had great influence in enzyme activity. The enzyme showed variable degradation effects on different types of dextran and its derivatives.

These results suggest that the dextranase secreted by *Bacillus subtilis* NRC-B233 is industrially important from the perspectives of its activity at across pH range (5.0–10.0), its thermo-activity in addition to its halophilic character and its ability to degrade different types of α -1,4 and α-1,6 glycosidic linkages.

Keywords: Halophilic dextranase, *Bacillus subtilis*, Dextranase stability.

Honey is a reservoir for microbes that withstand concentrated sugar levels , acidity, as well as also it has antimicrobial characteristics. The osmophilic feature of the honey could hypothesize the presence of moderate halophilic bacteria with new properties. Recently, a considerable attention has been given

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to the enzymes produced by moderately halophilic microorganisms and their biotechnological potentials (Ventosa *et al.,* 1998, Xue and *et al.,* 2008). Enzymes with optimal activity at high-salt concentrations are useful for many harsh industrial processes, where concentrated salt solutions otherwise inhibit many enzymatic conversions (Amoozegar *et al.,* 2003; Hutcheon *et al.,* 2005 and Oren *et al.,* 2005). Halophilic enzymes perform the same enzyme function as their non halophilic counterparts, but they have substantially different properties. Among these are their requirements for high-salt concentrations in the 1–4 M range for activity and stability. Extremely and moderately halophilic microorganisms have been well described, but the apparent thermostable properties of their enzymes especially from moderately halophilic bacteria, have not **(**Tokunaga *et al.,* 2004**).**

Dextran is a collective name for high-molecular-weight polymers composed of D-glucose units connected with α-1,6 linkages and various amounts of side branches linked with α -1,2, α -1,3, or α-1,4 to the main chains. Dextranase (EC 3.2.1.11; _-1, 6-glucan 6-glucanohydro- hydrolyzes the 1-6 glycosidic linkage in dextran (Sankpal *et al.,* 2001). The enzyme cleaves the linkages of the dextran molecule and releases shorter isomaltosaccharides. It occurs in a variety of bacteria , *e.g: Bacillus subtilis* and *B.megaterium* (Hayward & Sly,1976). In the sugar-processing industry, contamination by dextran causes an extensive problem by increasing the viscosity of the sugar juice. The use of dextranase in the processing, however, reduces the viscosity (Clarke, 1997). For dental and industrial uses, it is necessary to use a dextranase which is stable and has an optimum pH range of neutral to alkaline. A large number of industrial processes in the environmental industry and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for such enzymes. Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources. Among the several factors that are important for microbial growth and enzyme production using a particular substrate are particle size and moisture level/water activity (Pandey, 1992, 1994).

The aim of the present study was to examine the production of dextranase under SSF using *Bacillus subtilis* isolated from honey and to optimize the fermentation medium for maximum enzyme activity. Furthermore, partial purification and characterization of dextranase were investigated in our research.

Materials and Methods

Isolation of bacterial strain from honey

The isolate NRC-B233 was isolated from honey produced in Saudi Arabia. One hundred microliters of honey was spread on solid agar plates (g/l): dextran, 10; $MgSO_4$, 0.02; K_2HPO_4, 5.5 and agar, 25. After drying for 20 min in a laminar flow hood , the plates were incubated at 50°C for 24 hr or until the bacterial

colonies were of a sufficient size for colony replication (approximately larger than 3-5 mm in diameter). The bacterial isolates were streaked onto fresh agar plates and preserved at 4°C. The purity of the isolate was assessed by colony morphology and microscopy.

Identification of the isolate

The isolate was identified as a novel *Bacillus subtilis* NRC-B233 on the base of 16S rRNA sequence and 16-23S intergenic region (Lopez & Alippi, 2007; Rodriguez *et al.*, 2007 and Shah *et al.,* 2007). DNA extraction and PCR amplification of 156srDNA region. DNA was isolated from the selected isolates coded KNRC1410 according to Sambrook *et al.* (1989). The 16srDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16srDNA region. The forward primer was 5'AGAGTTTGATCMTGGCTCAG3' and the reverse primer was 5'TACGGYTACCTTGTTACGACTT3'. The PCR mixture consists of 30 picomoles of each primer, 10ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis (Ausuble *et al.,* 1999) (Fig.1) and the remnant was purified using QIAquick PCR purification reagents (Qiagen). DNA sequences were obtained using an 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan), BigDye Terminator Cycle Sequencing (see details below). The PCR product was sequenced using the PCR primers described above. Blast program was used to asses the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

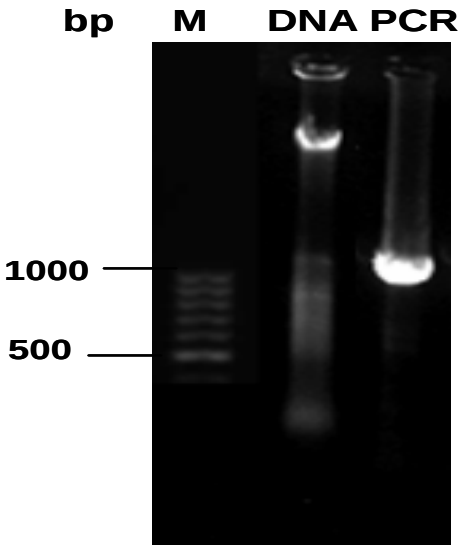

Fig. 1. Agarose gel electrophores.

DNA sequencing

Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger *et al.* (1977), was done using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan). The sequencing reaction was performed with four different fluorescent labels identifying the ddNTPs, instead of the radioactive labels. These flurophores were excited with two argon lasers at 488 and 514 nm, respectively when the respective bands passed the lasers during the electrophoresis. The specific emissions were detected and the data were collected for analysis (Prober *et al.,* 1987 and Freeman *et al.,* 1990). The thermal cycling mixture was as follows: 8 µl of BigDye terminator mix, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product or plasmid), then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95°C, then 49 cycles of 30 sec at 95°C, 10 sec at 52°C and 4min at 60°C. The products were purified using special column according to the instruction of the manufacturer. The elute were taken and add high dye formamide with (1:1)/volume ratio, run at 95ºC for 5 min for denaturation, shocked on ice. The sample sequenced in 3130 X DNA sequencer and analysised.

Chromosomal DNA and plasmid extraction

Chromosomal DNA was prepared from overnight culture in LB (in full at first mention), using AxyGEN BIOSCIENCES DNA extraction kit, according to Manufacturer's Instructions. Plasmid extraction was performed using Wizard mini prep. extraction kit (Promega) according to the manufacturer's instructions with slight modification, briefly 50 µl of lysozyme (200 mg/ml) was added to the resuspended buffer and incubated at 37°C for 1 hr then the protocol was carried on as described in the kit.

PCR amplification for molecular identification

To amplify the 16S rRNA gene, a primer pair hybridizing to two conserved regions in 16S rRNA genes from *Bacillus* spp. was used: (bac-F and bac-R) (Ash *et al.,* 1991). For the amplification of the 16–23S intergenic region, a primer pair was used: L516SF and L523SR.

Dextranase production media

The medium used to test the dextranase production had the following composition (g/l): dextran, 10; yeast extract, 2.5; $MgSO_4$, 0.02; K_2HPO_4, 5.5 (each separately autoclaved).

Cellular production

Cultivation was carried out in 250-ml Erlenmeyer flasks. Each flask contained 50 ml of production medium and was sterilized for 15 min at 121°C. The flasks were then inoculated with 2.0 ml spore suspention and incubated in a static incubator for 24 hr at 30°C. The culture broth was then centrifuged in a refrigerated centrifuge (K70; Janektzki, Germany) at 10,397g to separate the bacterial cells from the culture medium. Each fermentation run was performed in

triplicate, and analyses were carried out in duplicate. The data given are the means of all the measurements. The mean standard error of the dextranase estimate was f 0-25 U and ranged from f0.002 to f ±3.997.

Solid- state experiment

Ten grams of each substrate in 250 Erlenmeyer flasks (taro crust-, apple crust,- banana crust-, onion crust-, potato crust -, egg plant crust-, watermelon-rind, sawdust-, corn flour-, wheat flour-, commercial starch-, wheat bran or ground rice) were mixed with 8 ml of the production medium together with 10 ml of distilled water, mixed thoroughly and autoclaved at 121ºC for 30 min. They were cooled at room temperature and each flask was inoculated with 2 ml inoculum.

Extraction and enzyme recovery

Dextranase was extracted from the substrate using distilled water as the extracting agent (Balch *et al.*, 1979). Ten volumes of distilled water per gram substrate (based on initial dry weight of the substrate) was added to the fermented medium and the extraction was performed by agitation at room temperature in a rotary shaker for 60 min at 150 rpm. The slurry was then squeezed through Sun dried muslin cloth previously washed in distilled water and clarified by centrifugation at 5,000 rpm at 4ºC for 15 min. The clear supernatant was used to assay for the enzyme activity and protein content.

Assay of enzyme activity

The activity was measured using the Somogi-Nelson method (Somogi, 1952) with dextran (Mol.Wt.250000) as substrate; 0.3 ml of the filtrate medium was incubated with 0.7 ml of 2.5% dextrane (Mol.Wt.250000) in 0.1 M sodium citrate buffer at pH 5.2 and 50^{o}C for 15 min. Then 0.25 ml was taken for the assay.

One unit (U) of enzyme was defined as the amount of enzyme which liberated 1 µg of glucose equivalent in one min. Amount of protein was determined by the method of (Lowry *et al.*, 1956).

Chromatography

Paper chromatography was performed according to Block *et al.* (1995). Hydrolytic products from dextran (Mol.Wt.250000) were analyzed by paper chromatography on whatman No.1. The reaction mixtures at the end of incubation time were boiled for 3min to stop the reaction. Chromatographic development was carried out with a solvent system of n-butanol: acetone: water (4:5:1) and detected by spraying with aniline hydrogen phthalate.

Optimization of culture conditions

All the following experiments were conducted under SSF in the presence of corn flour as the best substrate.

The influence of temperature on growth and production of dextranase was studied at 25^{o}C, 30^{o}C, 37^{o}C, 50^{o}C and 60^{o}C. The effect of the incubation period was studied at (8, 24, 32, 48, 56 and 72 hr), at 37^{o}C. Effect of moisture content

was achieved using different volumes of distilled water (50, 100, 150, 200, 250, 300 and 500% (v/w)) at 37ºC. Dextranase production was also investigated with the initial pH adjusted to 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2 and 10.2 at 37ºC.

Nutrient additives

Nutrient additives included NaCl (5-35 g/l**)** and 0.1 M **(** $FeSO_4$, EDTA, $MnCl_2$, NaH_2PO_4, , $FeCl_3$, $LiSO_4$, $MgSO_4$, $K_2Cr_2O_7$, KI , K_2HPO_4 , Na_2HAsO_4 , $ZnSO_4$, $K_2S_2O_8$, KH_2PO_4 ,$CaCl_2$, KCl , $AlCl_3$, $CuSO_4$ and $NaCO_3$) with each salt added separately in the production medium. Also (5g/l) of the different organic nitrogen sources; urea, beef extract, yeast extract, ammonium chloride, peptone, casein and corn steep were used. In addition, different carbon sources ; (5g/l) (manose, glucose, galactose, sucrose, lactose, arabinose, cellulose, dextrane, xylose, starch and dextrin) were added in the medium to study their effects on dextranase activity.

Storage stability

The culture filtrate of the crude dextranase enzyme was stored in distilled water at 4°C for four months. The activity was measured every two weeks using 3.5 mg of protein.

Partial purification of dextranase

The ultra-filtration and fractional precipitation with acetone techniques were used. The first step of purification was carried out by passing 2 liters of crude enzyme through a Pellicon Cassette system with a membrane PLGC Cassette 10.000 NMWL. Low protein binding . The retentate and permeate obtained were checked for dextranase activity. The permeate fraction exhibited a dextranase activity.

Fractional precipitation using different concentrations (30-80%) of acetone by (Fukomoto, 1963) was used. The method was performed as follows: 100 ml permeate fraction was precipitated using 30 % v/v solvent and centrifuged at 2850 g for 10 min in a cooling centrifuge, (fraction 1) then 40 % v/v solvent was used to precipitate the supernatant (fraction 2). This method was repeated to reach to 80% v/v solvent concentration and the fraction that contained the highest enzyme activity was chosen for further experiments.

Effect of pH on partially purified dextranase

Enzyme activity was studied over the pH range 3.2-10.2.

Influence of incubation temperature

In this experiment, 0.3 ml of partially purified enzyme was incubated with 0.7 ml of 2.5% dextrane and incubated at different temperatures (35-75ºC) for 15 min.

Thermal stability

Profile was studied by incubating the enzyme preparation at various temperatures, (40-75°C) in glycine NaOH buffer 0.05M, at pH 10 for different incubation period (15-60min) and the residual activity was measured at 70ºC.

The pH stability of dextranase enzyme was examined by incubating the enzyme at room temperature at 0.5, 1, 1.5, 2 hr and different pH values 5.2 and 6.2 of (0.1 M) sodium – citrate buffer , 7.2 and 8.2 of (0.2 M) tris - buffer and 9.2 and 10.2 of (0.2 M) glycine – NaOH buffer.

The residual activity was measured as mentioned previously.

Influence of salts on enzyme activity

One ml of partially purified enzyme was dissolved in 1 ml of 0.1 M of the following salts: $MnCl_2$, $CrCl_3$, EDTA, $CaCl_2$, $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $AlCl_3.6H_2O$,$CaCO_3$, KCl and $ZnSO_4.7H_2O$, then incubated with the substrate and the activity was measured.

Effect of different concentrations of NaCl

One ml of partially purified enzyme was dissolved in 1 ml of different concentrations (1-15%) NaCl and incubated at room temperature for one hour. The enzyme activity was determined as described previously**.**

Effect of enzyme on different molecular weights of dextrans

Partially purified enzyme was used to degrade dextrans of different molecular weights (250 000 and 275000) blue dextran (2000) and sephadex G-100(1000-10000). Also some carbohydrates such as starch, amylopectin, cellulose, maltose and inulin were used .

Results

Characteristics of dextranase producing bacteria

The cells isolated from Saudi Arabian kashmiry honey were rod shaped Gram positive, motile and spore forming. Colonies were circular and cream, no pigment formed. The isolate was subjected to molecular identification based on 16S rRNA gene sequencing method (Fig.1). The 16S rRNA gene sequence analysis indicated that the isolate was *Bacillus* sp. with 98% identity to any of these three species *Bacillus spp.*, or *B. licheniformis*, or *B. subtilis* and they clustered into a monophyletic line in a phylogenetic tree.

To identify the isolate to a definite species the analogical electrophoresis, using NEB cutter was applied to identify the 16S rRNA results, which have been sequenced. The isolate showed different *AluI* fragments which differ to the *AluI* fragments generated from 16S rRNA sequence of *B. subtilis*, or *B. licheniformis* or *B. amyloliquefaciens.* It was clear that 16S rRNA gene alone could not distinguish this isolate.

Identification by 16-23S intergenic region

For complete identification of this isolate the 16–23S intergenic region was amplified with the primers L516SF X L523SR, then the sequence was determined for the isolate. The homology results for the 16–23S intergenic region reported that: the isolate NRC-B233 was 100% identical to *B. subtilis* .

Dextranase production and the effect of different parameters

The preliminary experiment using the shack culture technique and dextran as a substrate revealed that *Bacillus subtilis* NRC-B233 exhibited high dextrane degradation (258 Ulml), so it was chosen for this study. The chromatography of the compounds produced by incubating dextran (250000) with the crude enzyme suggested a random endo-type hydrolysis resulting in liberation of long-chain oligomers together with glucose and isomaltose units. Accordingly, different types of wastes products and cheap materials were applied under SSF using shaken and static conditions. Dextranase activity was produced in the presence of corn flour, – wheat flour – wheat bran, –commercial starch, – grinded rice (Fig. 2). The highest activity was achieved using corn flour (75.276, 61.323 U/g) shaken and static, respectively. The optimum temperature for maximum enzyme production was obtained at 37^{o}C (140.177, 108.192 U/ml shaken and static, respectively, below and above this temperature there was a clear decrease in enzyme production (Fig. 3). The difference between the shaking and static was significant. Consequently the cultures in the following experiments were incubated statically in order to save energy. The enzyme activity was increased by increasing the time of incubation to reach the maximum value (158.961U/g) after 32 hr. There was a gradual decrease in enzyme activity between (48 and 72 hr) (Fig. 4). The optimum dextranase production (170.624 U/g corn flour) was observed at 200% (v/w) moisture level (Fig. 5). *Bacillus subtilis* produced dextranase across pH range from 3 to 11 and the maximum enzyme production was obtained at pH10 (216.134 U/g) (Fig. 6). The addition of NaCl (5-20 g/l) had no influence on enzyme production. Concentrations higher than 20g/l reduced enzyme production gradually and at 35 g/l NaCl enzyme activity was reduced by 55% (Fig. 7). The influence of different salts (0.1M) on dextranase production had variable effects on dextranase production. The production increased in the presence of KCl and $AlCl_3$ with the maximum activity in the presence of $CrCl_3$ (447.580 U/g). The influence of different concentrations of $CrCl_3$ (0.025- 2M) on the enzyme activity showed that a gradual increase in activity was obtained by increasing the concentration of $CrCl_3$ and 0.175 M was the optimum for enzyme productivity (Fig. 8b). Different organic nitrogen sources (5g/l) were separately added to the fermentation media to evaluate their effects on dextranase activity and (5g/l) casein, ammonium chloride, yeast extract and corn steep increased its activity (Fig. 9a). The maximum activity (1076.768 U/g) was obtained when (5g/l) peptone was added. On the other hand, urea, malt extract and beef extract decreased the activity compared to the control (without nitrogen source).

2% peptone provided the best dextranase activity (1225 U/g) (Fig. 9b). Whilst the best carbon source was (5g/l) starch producing a dextranase activity of 1553.364U/g (Fig. 10), whilst, dextrin, mannose and fructose reduced the enzyme production to 67%, 84% and 75%, respectively.

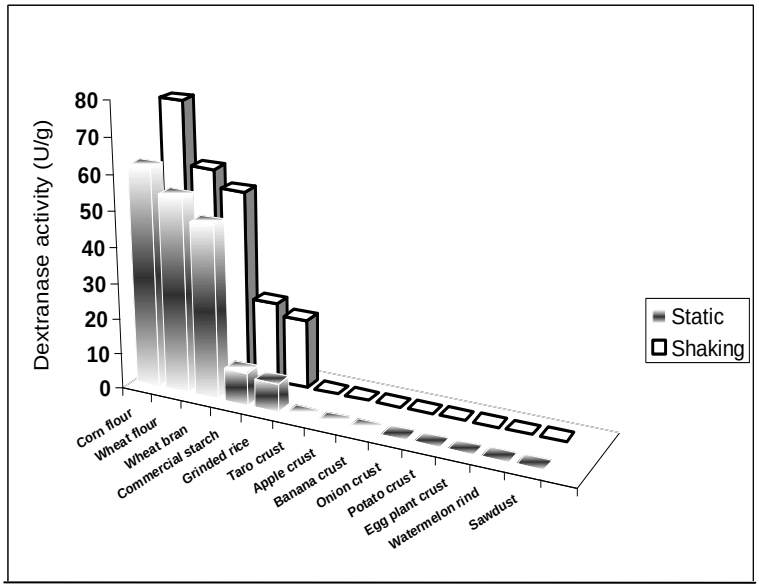

Fig. 2. Effect of different wastes and commercial materials on dextranase production from *Bacillus subtilis* KNRC.

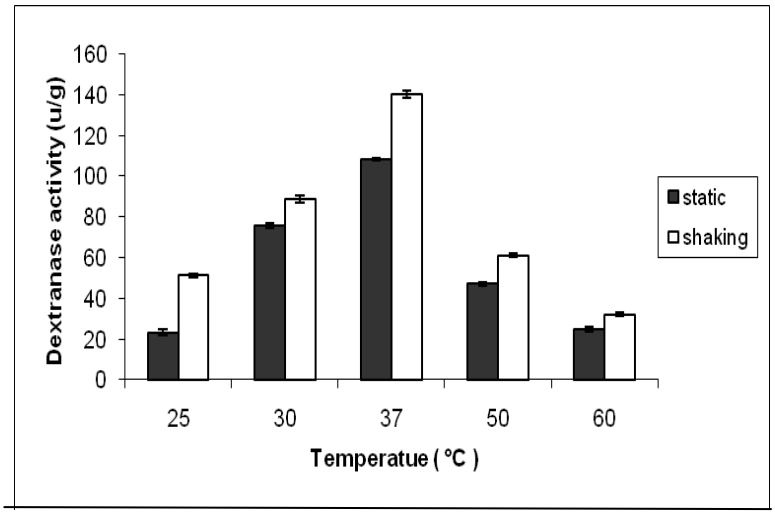

Fig. 3. Effect of different temperature on dextranse production from *Bacillus subtilis* KNR.

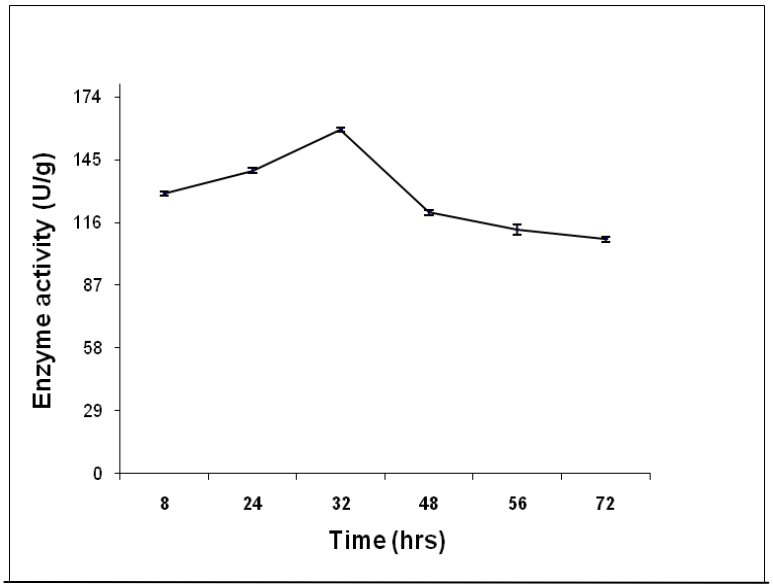

Fig. 4. Effect of incubation time on dextranase production from *Bacillus subtilis* KNRC.

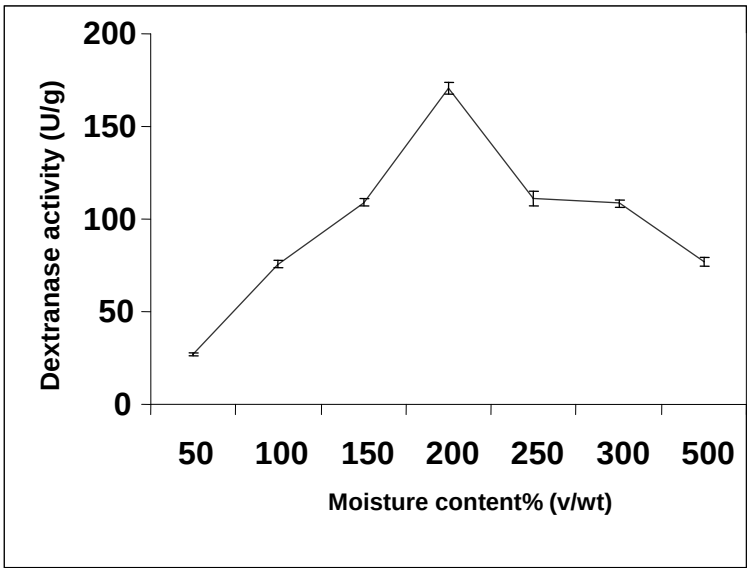

Fig. 5. Effect of different moisture content on dextranse production from *Bacillus subtilis* KNRC.

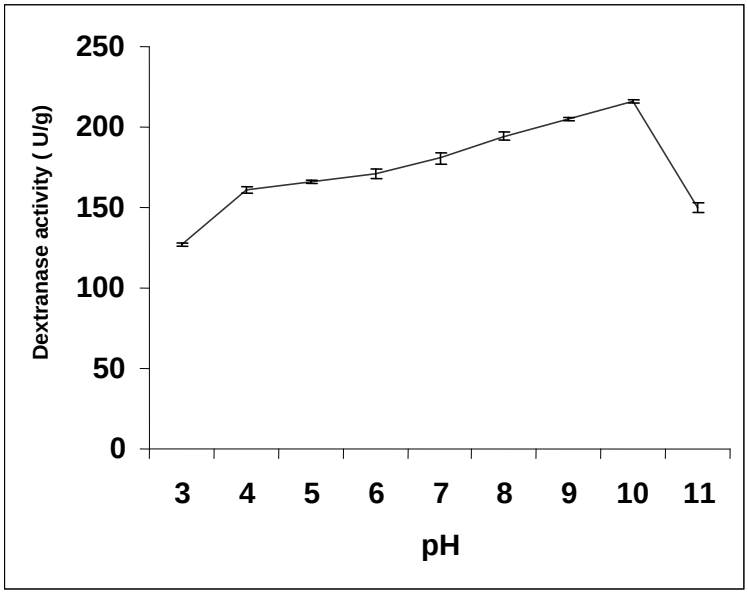

Fig. 6. Effect of different pH on dextranse production from *Bacillus subtilis* KNRC.

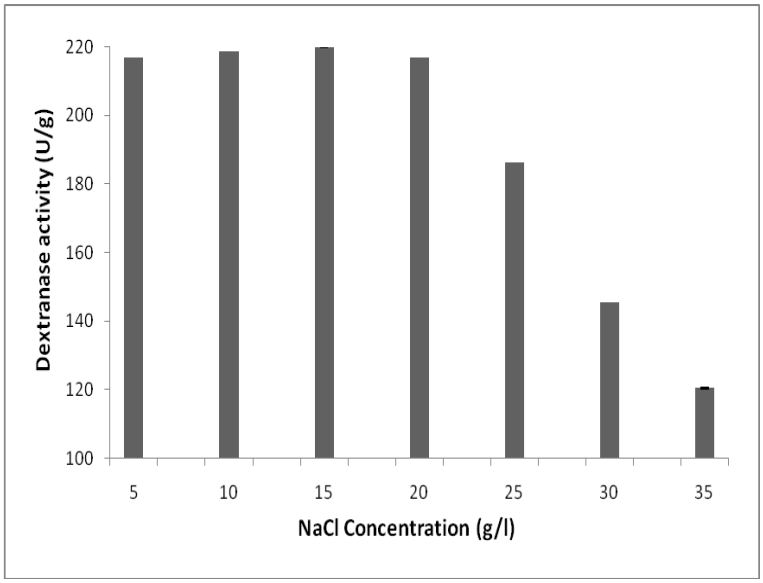

Fig. 7. Effect of different NaCl concentations on dextranase activity from *Bacillus subtilis* KNRC.

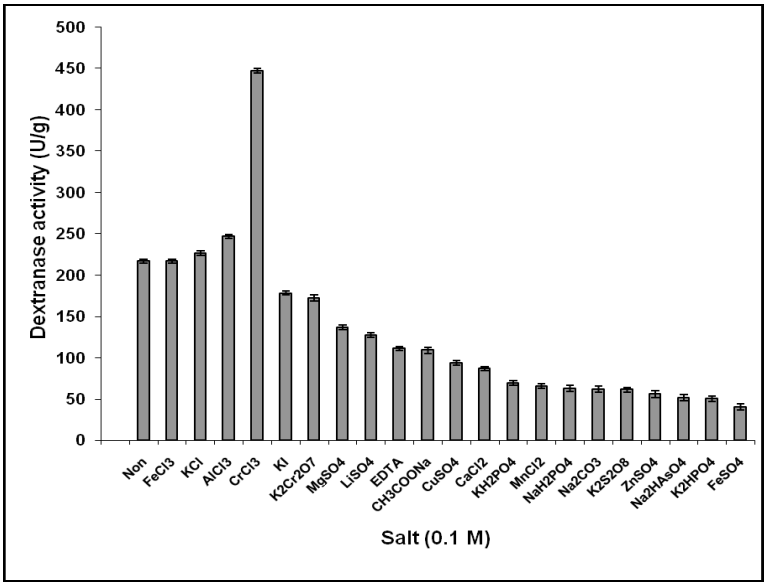

Fig. 8a. Effect of different salts on dextranase production from *Bacillus subtilis* KNRC.

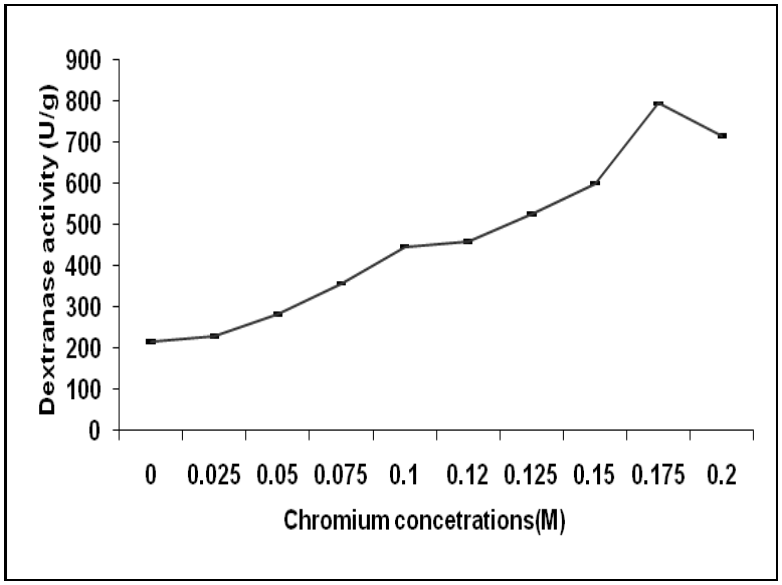

Fig. 8b. Effect of different concentrations of Chromium chloride on dextranase production from *Bacillus subtilis* NRC-B233.

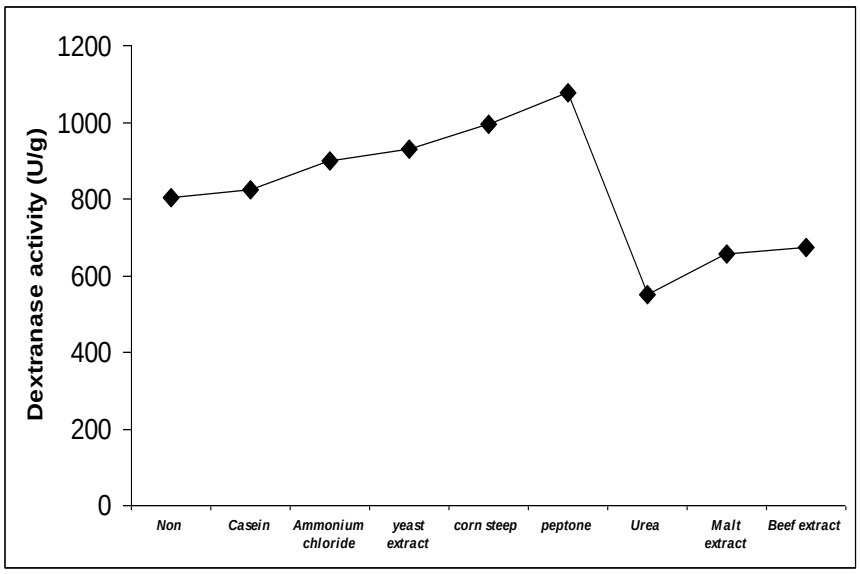

Fig. 9a. Effect of different nitrogen sources on dextranse production from *Bacillus subtilis* KNRC.

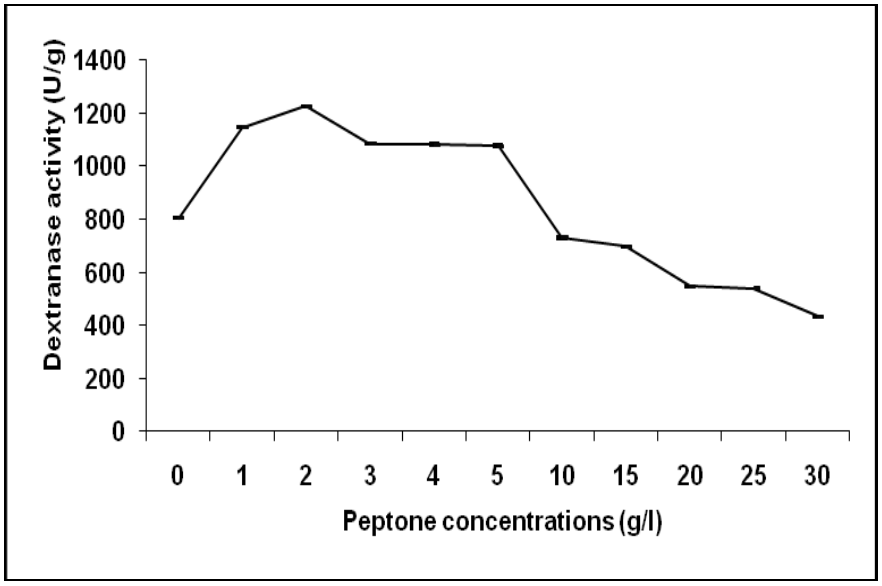

Fig. 9b. Effect of different peptone concentrations on dextranse production from *Bacillus subtilis* KNRC.

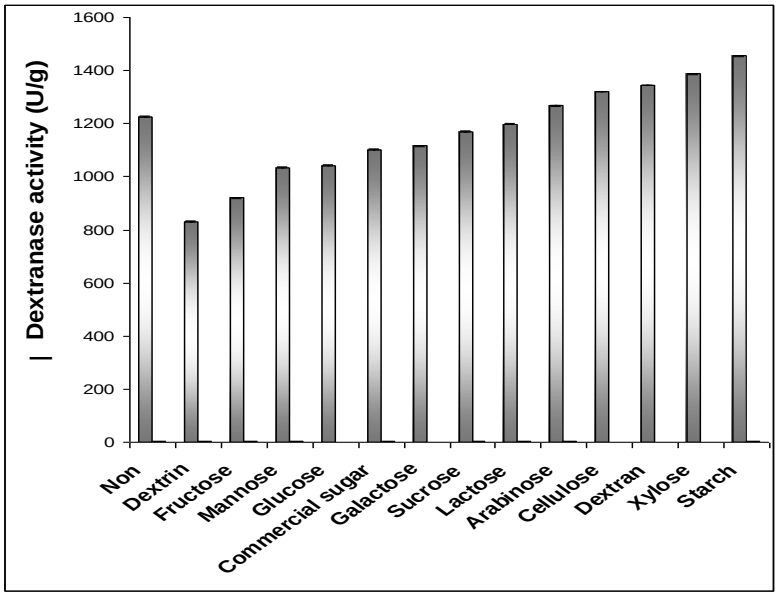

Fig. 10. Effect of different carbon sources on dextranse production from *Bacillus subtilis* KNRC.

Partial purification of dextranase

Ultrafilteration of the culture broth using membrane PLGC Cassette10.000 NMWL.resulted in 84.2% dextranase recovery. The result indicated that dextranase aggregated on low molecular weight cutoff membranes. The concomitant recovery of dextranase using 70 % acetone showed a recovery of about 37.3% (Table 1).

TABLE 1. Partial purification steps of *Bacillus subtilis* KNRC dextranase.

Purification steps	Total activity (U)	Recovered activity (%)	Total protein (mg)	Recovered protein (%)	Specific activity (U/mg)	Fold of purification
Culture filtrate	249931	100.0	4477.4	100.0	55.8	1
Ultra filtration	201446	84.2	1276.5	28.5	164.9	2.96
Acetone (70 %)	78424	37.3	12.525	0.0028	6261	112.2

Effect of different parameters on dextranase activity

The results in Fig. 11 and 12 showed that alkaline media and high temperatures were the most favorable for the enzyme activity. Maximum activity was obtained at pH 9.2 and 70^{o}C. A sharp decrease in dextranase was noticed at acidic pH (3.2 and 4.2) and at 35^{o}C. The thermal stability of the partially purified dextranase showed

that the enzyme was fairly stable to heat treatment in absence of the substrate (Table 2). At 75 ºC the enzyme retained its complete stability for one hour. The pH of the enzyme exhibited stability pH across wide range of pH. The enzyme exhibited maximum stability at pH 10.2 after half hour (Table 3). The effect of different salts (Fig. 13) showed that NaCl from (1-15%) played a significant role in enzyme activity where the activity at 10% increased about four-fold. On the other hand, EDTA, $CaCl_2$, KCl and Mg_2SO_4 all enhanced enzyme activation (Table 4). Whereas dextranase activity was strongly inhibited by $MnCl_2$ and $AlCl_3$ and the retained activities were 31.3%, 36.9%, respectively. The substrate specificity of *Bacillus subtilis* KNRC on different types of carbohydrates and the rate of enzyme hydrolysis with various glycosidic linkages was done. Blue dextran (1000M.wt) gave the maximum relative enzyme activity (273.8 %) (Table 5). Substantial dextranolytic activity was found when starch (α -1,4 and α-1,6 glycosidic linkages), amylopectin, maltose, inulin and cellulose (α -1,4 glycosidic linkages) were used as carbon sources. The crude enzyme was highly tolerant to repeated freezing and thawing, the activity remaining at 100% for three months.

TABLE 2. Thermal stability of *Bacillus subtilis* KNRC dextranase.

Temperature (°C)	Exposure time (min)			
	30	60	90	120
40	100 ± 0.031	100 ± 0.029	100 ± 0.066	100 ± 0.092
45	100 ± 0.016	100 ± 0.017	100 ± 0.029	100 ± 0.008
* 50	100 ± 0.003	100 ± 0.034	100 ± 0.033	100 ± 0.049
55	100 ± 0.022	100 ± 0.021	100 ± 0.050	100 ± 0.053
60	100 ± 0.020	100 ± 0.019	100 ± 0.070	100 ± 0.030
65	100 ± 0.046	100 ± 0.080	100 ± 0.090	100 ± 0.027
70	100 ± 0.060	100 ± 0.005	100 ± 0.037	100 ± 0.025
75	100 ± 0.009	100 ± 0.034	80.6 ± 0.030	80.6 ± 0.057

Relative values of dextranase activity of enzyme, expressed in % and obtained by Berridge's method
* Control (The original temperature)

TABLE 3. pH stability of the partially purified *Bacillus subtilis* KNRC dextranase .

pH	Exposure time (min)			
	30	60	90	120
5.20*	100 ± 0.031	100 ± 0.029	90 ± 0.066	83 ± 0.092
6.20	100 ± 0.016	100 ± 0.017	100 ± 0.029	100 ± 0.008
7.2	100 ± 0.003	100 ± 0.034	100 ± 0.033	100 ± 0.049
8.2	100 ± 0.022	100 ± 0.021	100 ± 0.050	100 ± 0.053
9.2	100 ± 0.020	100 ± 0.019	100 ± 0.070	100 ± 0.030
10.2	100 ± 0.046	95 ± 0.080	81 ± 0.090	80 ± 0.027

Relative values of dextranase activity of enzyme, expressed in % and obtained by Berridge's method.
* Control (The origonal temperature)

TABLE 4. Effect of different salts on *Bacillus subtilis* K dextranase activity.

Salt (0.1 M)	**Relative activity (%)**
*Non	100 ±0.008
$MnCl_2$	31.3 ±0.001
$CrCl_3$	125 ±0.031
EDTA	264.4 ±0.053
$CaCl_2$	228.8 ±0.22
$MgSO_4$	181.25 ±0.029
NaCl	104.9 ±0.024
$CuSO_4$	77.5 ±0.064
$AlCl_3$	36.9 ±0.006
$CaCO_3$	100 ±0.027
KCl	207.6 ±0.015
$ZnSO_4$	84.5 ±0.019

* Control (without any salt)

TABLE 5. Action of *Bacillus subtilis* NRCK dextranase on various carbohydrates.

Dextran type	**Main linkage**	**Relative activity (%)**
Dextran * (250.000M.wt)	α-1,6	100
Dextran (200.000-275.000 M.wt)	α-1,6	79.2
Blue dextran (2000 M.wt)	α-1,6	273.8
Sephadex G-100 (1000-10.000M.wt)	α-1,6	112
Starch	α-1,6	228
Amylopectin	α-1,6	165
Maltose	α-1,4	71
Cellulose	α-1,4	100
Inulin	α-1,4	170

*Control

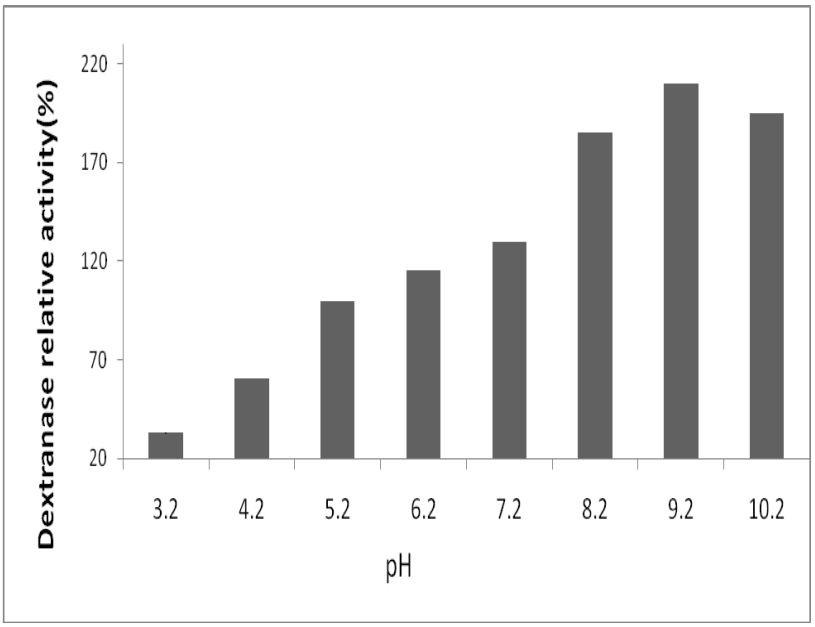

Fig. 11. Effect of different pH on partial purified dextranse.

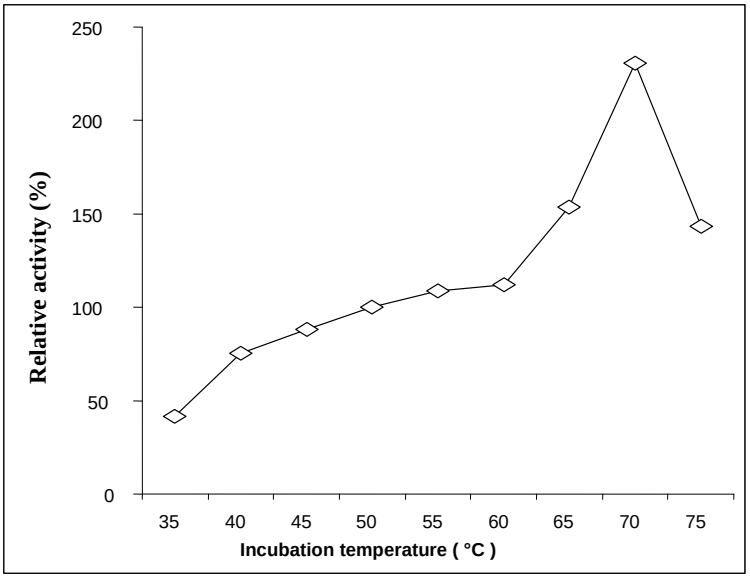

Fig. 12. Effect of different temperatures on partial purified dextranse.

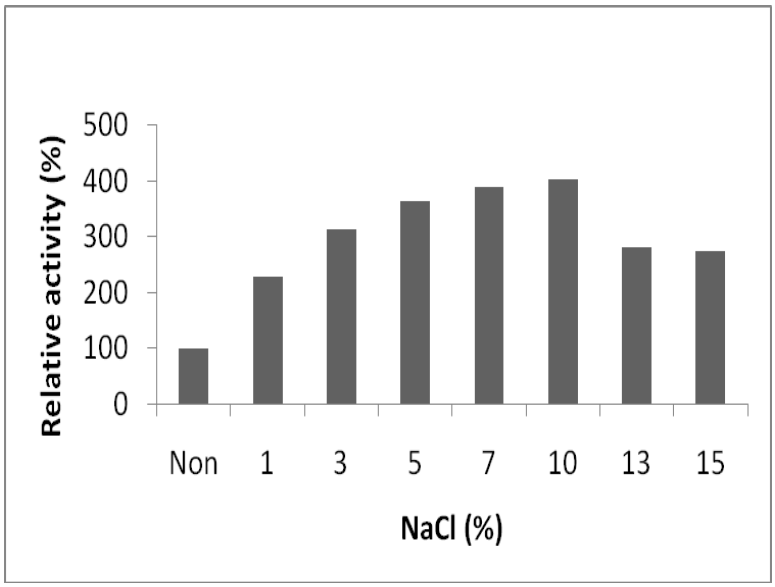

Fig. 13. Effect of different NaCl concentrations on partial purified dextranse.

Discussion

The *Bacillus subtilis* isolate (NRC-B233) obtained from Saudia Arabian honey was shown to have substantial potential as a dextranase producer in a range of substrates, temperatures and pH levels. It was selected for further studies since it appeared to be the best producer of extracellular dextranase. The chromatography products from dextran MT 250000 with crude enzyme suggested a random endo-type hydrolysis resulting in the liberation of long-chain oligomers together with glucose and isomaltose units. Unique characters of this isolate was its ability to produce dextranase in the absence and presence of NaCl (5-25 g/l) without a reduction in enzyme production. In contrast, Abdel - Naby *et al.* (1999) reported that using of starch instead of dextran led to a drastic decrease in enzyme production. They showed that the major regulatory mechanism of dextranase is constitutive. The mutant *Lipomyces starkeyi* produced constitutive dextranase when grown on glucose, fructose and sucrose as well as on dextran (Kim & Day, 1995). In the present study, enzyme productivity was significantly affected by temperature and pH, with the optimum enzyme production was at 37^{o}C and pH 9. Similar results were reported by Yamsguchi & Gocho (1973) since *Brevibacterium* dextranase had an optimum dextranase activity at pH 8.0 and 37°C. In contrast, Bhatia *et al.* (2010) showed that maximum dextranase production by *Paecilomyces lilacinus* was achieved at pH 6.0 and at temperature 30°C. In the present study, the most potent activity of the enzyme was noticed at 200% (v/wt) moisture level. Whilst nitrogen sources including organic and inorganic nitrogen showed that corn steep and peptone were the most effective for enzyme production. Yamsguchi & Gocho (1973),

reported that polypeptone was the most effective nitrogen source for *Brevibacterium* dextranase production among the compounds tested. $CrCl_3$ increased enzyme productivity by more than two-fold. $CrCl_3$ could play an important role as a dextranase stimulator.

The use of ultrafilteration for downstream processing would result in a one-step, cost-effective method of recovery for dextranase. The molecular weight of the partially purified enzyme was less than 10 KDa. The halophilic bacteria's enzymes are thus active at salt concentrations which inhibit or even denature many enzymes of non-halophilic organisms. This must be reflected in marked differences in the composition and properties of the protein molecules (Norberg & Hofsten, 1969). It was suggested that the halophilic enzymes have smaller molecular weight than most other enzymes, which would make them more resistant to being 'salted' out. However, no halophilic dextranase have yet been studied in sufficient detail to give substantial support to this hypothesis.

The properties of the partially purified enzyme clearly showed that high temperature and alkaline media were the most favorable for the enzyme activity. The enzyme showed optimal activity at 70°C, indicating that an increase in activity with temperature offsets the thermal denaturation. It was reported that the optimal temperature for *Thermotoga lettingae* TMO dextranse was 55–60°C during 15 min incubation (Kim & Kim, 2010). The optimum pH for dextranase activity was 9. In contrast Wynter *et al.* (1996) reported that ammonium sulfate precipitated crude dextranase with its optimum pH between 5 and 6. The use of ultrafilteration for downstream processing would result in a one-step, cost-effective method of recovery. The partially purified dextranase was greatly affected by the addition of metal ions, the halophilic feature of this enzyme appeared clearly, when the enzyme activity increased about four-fold in the presence of 10% NaCl. Thus, the effects of salt concentration on the efficiency of dextranase productin should be an interesting study to conduct in the future. Sugiura et al., (1973) reported that dextranase from *Penicillium funiculosum* was activated by Co^{2+}, Mn^{2+} and Cu^{2+} and inactivated by Ag^{+}, Hg^{2+}, *N*-bromosuccinimide and iodine. In contrast, *Thermotoga lettingae* TMO dextranase activity was not significantly affected by the presence of metal ions, except for the strong inhibition by 1 mM Fe^{2+} and Ag^{2+} (Kim & Kim, 2010). The enzyme showed complete stability at 75 °C till one hour. In contrast, the alkaline dextranase from *Brevibacterium* was stable at temperatures below 60°C (Yamsguchi & Gocho, 1973). An attractive industrial application of thermostable dextranase is sugar processing. *Penicillium* sp. and *Chaetomium gracile* dextranases that are active and stable above 55°C would improve processing of dextran-contaminated cane juice in the sugar industry (Khalikova *et al.*, 2005). In the present study, the partially purified dextranse exhibited a broad pH stability range (pH 4.3–10.0), especially on the alkaline side. Similar results were reported by Kim & Kim (2010). The broad pH stability of our enzyme could be useful in sugar processing, which involves both acidic and alkaline conditions. This enzyme could act efficiently on high and low molecular weight dextranase. Where it could preferentially split a series of dextrans and their derivatives

(Sephadex), but in the case of dextrans, the rate of hydrolysis was dependent on the molecular weight of the substrate. Pleszczyńska *et al.* (1996) reported that *Penicillium notatum* dextranase which could act on different types of dextran and the rate of hydrolysis was independent of the molecular weight of substrate. The digestion products from the Sephadex derivatives showed the presence of reducing sugars. The previous result suggests dextranase to be an enzyme available for removal of dental cariogenic plaque deposits (Lifschitz & Bauer, 1976).

A great dextranolytic activity was found when starch, (α 1, 4 and α-1,6 glycosidic linkages) and amylopectin (α 1,4). Competition studies with different amounts of dextran and starch as substrates showed consistency with the hypothesis that hydrolysis of dextran and starch occurs at two independent active sites (Kim *et al.,* 2001., Lee *et al.,* 2003 and Ryu *et al.,* 2000). Degradation of alpha-linked D-gluco-oligosaccharides and dextrans by an isomalto-dextranase preparation from *Arthrobacter globiformis* T6 was reported by Torii *et al.,* (1976**)**.

Conclusion

In this study we focused on the isolation of halophilic bacteria from honey as a new source of bacteria producing dextranase. The honey isolate produced a novel halophilic low molecular weight constitutive dextranase characterized by unique features, like thermostability and pH stability. Further, cheap substrates like corn starch would be a superior alternative to the already available expensive dextran, since 30–40% of the production cost of industrial enzymes is accounted by the cost of the growth medium.

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إنتاج ديكسترانيز جديد محب للملوحة من باسيلاس ساتلس KNRC المتحور و المعزول من العسل تحت ظروف التخمر الصلب

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تم عزل *باسيلاس ساتلس* KNRC موجب صبغة جرام،متجرثم،محب للملوحة من عسل النحل المستورد من المملكة العربية السعودية. عرفت البكتيريا باستخدام 16sRNA و 16-23S. باستخدام العديد من النفايات تحت ظروف التخمر الصلب وجد أن دقيق الذرة كان الوسط الغذائى الملائم لأعلى إنتاجية من الدكسترانيز (61.323 U/g).

كانت أعلى إنتاجية للإنزيم محل الدراسة عند درجة حرارة ٣٧℃ ، pH 10، وفترة تحضين 23h و 200% نسبة ماء حيث وصلت إنتاجية الإنزيم (170.624 U/g).

عند استخدام العديد من مصادر الكربون والنيتروجين فى الوسط الغذائى للبكتيريا المنتجة للدكسترانيز وجد أن نشاطية الإنزيم قد وصلت إلى (1076.768 و 1553.364 U/g) عند استخدام 2% بيبتون و 5% نشا.

إن الصفة النادرة لهذه البكتيريا هى قدرتها على إنتاج الإنزيم باستخدام وسط غذائى عالى الملوحة (5-20 g/l) كلوريد الصوديوم.

إن إضافة 0.175 Mm من كلوريد الكروميوم قد أدى إلى مضاعفة النشاطية الإنزيمية أربع مرات ونصف.

عند استخدام الأشعة فوق البنفسجية لعمل تحور فى البكتيريا المنتجة للدكسترانيز وجد أن أعلى نشاطية للإنزيم كانت(2824.29) وذلك بعد 15 دقيقة من تعرض البكتيريا للأشعة.

لقد أعطى الإنزيم المنقى جزئيا أعلى نشاطية عند pH 9.2 و 70 C وقد زادت إنتاجية الإنزيم أ ربعة أضعاف بإضافة 10% كلوريد الصوديوم.

لقد أثبتت هذه الدراسة أهمية الإنزيم صناعيا وذلك لتحمله تركيزات ملوحة و درجات حرارة و حموضة وقاعدية عالية بالإضافة إلى قدرته على كسر الروابط من نوع α-1,4 and α-1,6 glycosidic linkages.