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Characterization of Extracellular Dextranase from a Novel Halophilic *Bacillus subtilis NRC-B233b* a Mutagenic Honey Isolate under Solid State Fermentation

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Abstract *Bacillus subtilis NRC-B233b* was isolated from Libyan honey sample proved to be a potent dextranase producer by applying solid state fermentation and utilizing corn flour as the sole carbon source. The optimized culture conditions for dextranase productions were 37°C, pH 10, 32 h, and 20% (v/w) moisture content. A unique character of this isolate is its ability to produce steady dextranase irrespective to the presence of NaCl in the medium. The addition of 0.175 Mm $CrCl_3$ increased the enzyme production by about 4.5 fold. Further improvement in enzyme production was achieved by simple UV mutation which increased the enzyme production up to about 2842 U/g. The crude extract has been partially purified about 112-fold from crude extract by only two purification steps involving ultra-filtration. The partially 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 4 fold in the presence of 10% NaCl. This enzyme showed variable degradation effect on different types of dextran and its derivatives. The treatment of viscous sugar cane juice with the enzyme preparation resulted in clear visual dextran hydrolysis. These results suggest that the dextranase produced by *Bacillus subtilis NRC-B233b* is industrially applicable.

Keywords: halophilic dextranase, thermophilic dextranase, *Bacillus subtilis*, dextranase stability.

Introduction

Honey can be considered as a reservoir for microbes that withstand the concentrated sugar, acidity. These microbes also contribute to the antimicrobial characters of honey. The

osmophilic feature of the honey could hypothesize the presence of moderate halophilic bacteria with new properties.

Recently, a considerable attention has been given to the enzymes produced by moderately halophilic microorganisms and their biotechnological potentials [1, 2]. Enzymes with optimal activity at high-salt concentrations are useful for many harsh industrial processes, where concentrated salt solutions otherwise inhibits many enzymatic conversions [3-5]. Halophilic enzymes perform the same enzyme function as their non halophilic counterparts, but they have substantially different properties. Among these, requirement for high-salt concentrations in 1–4 Molar range for activity and stability. In spite of the fact that many moderately and extremely halophilic microorganisms have been well described, the apparent thermostable properties of their enzymes, especially from moderately halophilic bacteria, have not studied extensively [6].

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 [7]. Dextranase (EC 3.2.1.11; α-1,6-glucan-6-glucanohydrolase) hydrolyzes the 1-6 glycosidic linkage in dextran chain [7]. The enzyme cleaves the linkages of the dextran molecule and releases shorter isomaltosaccharides. This enzyme was found in a variety of bacteria belong to *Bacillus* species such as: *B. subtilis* and *B. megaterium* [8]. In sugar-processing industries, contamination by dextran causes an extensive problem by increasing the viscosity of the sugar juice. The use of dextranase in sugar production process can reduces the viscosity significantly [9]. For many industrial applications, it is necessary to use a dextranase which is stable and has neutral to alkaline pH range. A large number of industrial processes in the areas of environmental industry and food biotechnology utilize enzymes at some stage or the other throughout the production process.

Of different processes for enzyme production, 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, particle size and moisture level/water activity are the most critical [10-12].

The aim of the present study is to examine the production of dextranase from honey isolate under SSF and to optimize the fermentation medium for maximum enzyme production. Mutation of the producer bacterium was one of our purposes to improve the production of the enzyme. Furthermore, partial purification, and characterization of dextranase were investigated.

Experimental

Isolation of Bacterial Strain from Honey

One hundred micro liters of honey (kashmiry honey: a honey bee collecting nectar from desert flower, Saudi Arabia) was spread on solid agar plates composed of (g/l): dextran, 10; $MgSO_4$, 0.02; K_2HPO_4, 5.5. After drying for 20 min in a laminar flow hood, the plates were incubated at 50 °C for 24 h. Among different strains isolated, the highest dextranase producer was further purified and characterized. The purity of the isolate was assessed by colony morphology and direct microscopic observation.

Identification of the Isolate

The isolated strains were examined by microscopic observation, and the selected culture was purified and tested for Gram's staining. Growth at different temperatures (25, 30, 40, 50, and

60°C), NaCl concentrations (0, 4, 8, 12, and 15%) and pH (4-11) were tested in nutrient broth. Identification was based on morphological, 16S rRNA sequence and biochemical tests (API). 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'. After full identification, the bacterium was deposited in NRC culture collection under the name *Bacillus subtilis NRC-B233b* [13-15]. Biochemical test (API) was achieved in the Holding Company for Biological Products and Vaccines (VACCERA), Cairo, Egypt.

Cell Banking

This strain was sub cultured in agar medium and the arisen colonies were harvested by glycerol solution (20%) and put in series of 2 ml. crygen vials (Nalgene, USA). The tubes were frozen immediately at -20 °C for 24 h followed by storage as working cell bank at -80 °C for further use. This was an important step to ensure that the starter culture of each experiments of the same generation number.

Dextranase production media

The medium used for dextranase production had the following composition (g/l): dextran, 10; yeast extract, 2.5; $MgSO_4$, 0.02; K_2HPO_4, 5.5 (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 minutes at 121ºC. The flasks were then inoculated with 2.0 ml spore suspention and incubated in a static incubator for 24 hours at 30 ºC. 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 (corn flour-, wheat flour- , commercial starch- , wheat bran or ground rice) were mixed with 8 ml of the production medium and autoclaved at 121 ºC for 30 min. Flasks were cooled at room temperature and each flask was inoculated with 2 ml of bacterial suspension (1 OD_{600}). All cultivations were performed in triplicate, and analyses were carried out in duplicate (i.e. the data given are the mean of six measurements).

Extraction and Enzyme Recovery

Dextranase was extracted from the substrate using distilled water as the extracting agent [17]. 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 4 ºC for 15 min. The clear supernatant was used to assay for the enzyme activity and protein content.

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 ^oC, 30 ^oC, 37 ^oC, 50 ^oC and 60 ^oC. The effect of the incubation period was

studied at (8, 24, 32, 48, 56 and 72 hrs), at 37 °C. Effect of moisture content was achieved using different volumes of distilled water (50, 100, 150, 200, 250, 300 and 500% (v/w) at 37 ºC. Dextranase production was also investigated with the initial pH adjusted to 3.2-11.2 at 37ºC.

Nutrient Additives

Nutrient additives included NaCl (0-15 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.

Determination of Enzyme Activity

The activity was measured using the Somogy-Nelson method using dextran of molecular weight 250 KDa as substrate [17]. Based on this method, 0.3 ml of the filtrate medium was incubated with 0.7 ml of 2.5% dextrane in 0.1 M sodium citrate buffer at pH 5.2 and 50°C. After incubation for 15 min, 0.25 ml was taken for analysis. One unit (U) of enzyme is defined as the amount of enzyme which liberates 1 µg of glucose equivalent in one min.
The amount of total protein was determined by the method of Lowry et al., (1956). This methods is based on the tyrosine and tryptophan content [18].

Sugar Determination by Chromatographic Method

Hydrolytic products of dextran were analyzed by paper chromatography and by HPLC method. Paper chromatography for sugar determination was performed according to Block et al., [19]. The reaction mixtures at the end of incubation time were boiled for 3 min to stop the enzymatic reaction. The obtained mixtures were plotted on chromatographic paper (Whatman 1). Chromatographic separation was carried out using a solvent system of n-butanol: acetone: water (4:5:1) and detected by spraying with aniline hydrogen phthalate. The acid hydrolysate of the hydrolytic product of dextran was analyzed using high-pressure liquid chromatography (HPLC). A 7.8×300-mm PL-HI-PLEXPB column linked to a differential refractometer was used for sugar analaysis. The column temperature was maintained at 80 ºC and the aqueous mobile phase was delivered at a flow rate of 0.6 ml/min. Same method was previously done by [20]

Storage Stability

The culture filtrate of the crude dextranase enzyme was stored in distilled water at 4°C for 4 months. The activity was measured every 2 weeks using 3.5 mg of protein as previously mentioned.

Mutation

Mutation of isolated bacterium *Bacillus subtilis NRC-B233b* from honey was performed according to the method of Abdel-aziz, et al., [21] with some modification: Twenty four hours old *Bacillus subtilis NRC-B233b* culture was cultivated in broth medium, and then suspended in 50 ml saline solution. Cell suspension was transferred to 14 cm diameter petri dishes. All samples were placed at distance of 20 cm from UV Lamp (40 W). Cell suspension was irradiated for 1 hr. One ml of treated culture was taken at different time intervals of 2 minutes. Treated culture were kept in dark for onehour before being diluted and spreaded onto thecomplete medium (CM). Irradiated cultures were maintained on nutrient agar containing the following components (g/L): glucose, 10, peptone 6, yeast extract 3, meat extract 1.5 and agar 28.

Repeated Batch

The freshly prepared mutagenic *Bacillus subtilis NRC-B233b* was cultivated under the optimized medium and conditions previously discussed, and then the enzyme activity was determined. Renew and cultivation for the mutagenic *Bacillus subtilis NRC-B233b* was repeated ten times and its dextranase activity was evaluated.

Partial Purification of Dextranase

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.

Fractional precipitation using different concentrations (30-80%) of acetone [22] 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 at different pH values 3.2, 4.2, 5,2 and 6,2 of (0.1 M) sodium – citrate buffer , 7, 2 and 8, 2 of (0.2 M) tris - buffer, 9.2, 10.2 of (0.2 M) glycine – NaOH buffer.

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 (35-75^{o}C) for 15 min.

Thermal Stability

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

The pH stability of dextranase enzyme was examined by incubating the enzyme at room temperature at 0.5, 1, 1.5, and 2 hours 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 and 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, maltose and inulin were used (Table 5).

Effect of Partial Pure Dextranase on Sugarcane Juice

Different concentrations of partial purified enzymes (237, 475, 712, 950 and 1185 U) were added to stored sugar cane juice of high viscosity (10 ml) and incubated at 70°C for 2 hours and the change in sugar juice viscosity was determined by a U-shapped glass Oswald viscometer at 30°C.

Results and Discussion

The adaptation and survival abilities of halophilic microorganisms in a wide range of salinities (0.5–25%) offer potential applications in various fields of biotechnology [23]. The osmophilic feature of honey suggests that it is being a good media for the presence of halophilic spore forming bacteria. Among these, the honey isolate *Bacillus subtilis NRC-B233b* was identified on the basis of morphological examination, 16S rRNA sequence and biochemical tests (API). The cells were rod shaped, Gram positive, motile and spore forming. Colonies were circular and cream, no pigment formed. The isolate grew optimally at 50ºC and pH 5. At 60ºC the growth was noticeably decreased. The culture grew well when the medium was supplemented with different concentrations of (NaCl 0-12%) but it did not grow at higher NaCl concentration (15% NaCl) and the maximum growth was observed at 4% NaCl. 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 *Alu*I fragments which differ to the *Alu*I fragments generated from 16S rRNA sequence of *B. subtilis*, or *B. licheniformis* or *B. amyloliquefaciens*. The API results confirmed that the isolate is belonging to *Bacillus subtilis*.

The isolate was selected for further studies since it appeared to be the best producer of extracellular dextranase. The preliminary experiment using dextran as substrate revealed that *Bacillus subtilis NRC-B233b* exhibited high dextranase production (258 U/ml) in liquid culture. The chromatography of the compounds produced by incubating dextran (250 kD) with the crude enzyme suggested a random endo-type hydrolysis resulting in liberation of long-chain oligomers together with glucose and isomaltose units. Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It has special interest in those processes where the crude fermented products could be used directly as enzyme sources [11]. Accordingly, different types of cheap starch rich materials were applied under SSF conditions. The results in Fig. 1 showed that dextranase was produced using corn flour, wheat flour, wheat bran, commercial starch and grinded rice as substrates. The highest enzyme activity (61.3 U/g) was achieved by using corn flour. Starch is known to be a slow release carbon source and a better substrate for enzyme production compared to simple sugars that lead to catabolite repression [24]. On contrary, Abdel-Naby et al., [25] reported that the use of starch led to drastic decrease in enzyme production. The previous results indicated 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 [26]. A unique character of this isolate was its ability to grow and produce dextranase in the absence and presence of NaCl (0-15 g/l) without reduction in enzyme production (data not shown). It could be concluded that the halophilic character of the osmphilic honey isolate not obligatory led to increase or decrease in enzyme production. On contrary, *Halobacillus* sp. MA-2 which was previously isolated from saline soil exhibited maximum amylase activity in the medium containing 5% (w/v) NaCl [3].

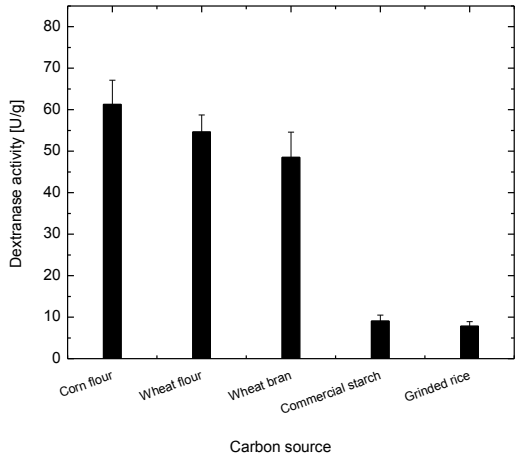

Figure 1. Effect of different starchy raw materials on dextranase production from *Bacillus subtilis NRC-B233b*.

The optimum temperature for maximum enzyme production was obtained at 37^{o}C (108.2 U/ml). Below and above this temperature there was a clear decrease in enzyme production (Fig 2). The enzyme activity was increased by increasing the time of incubation to reach the maximum value (158.9 U/g) after 32 hrs. There was a gradual decrease in the activity by increasing incubation periods from (48 to 72 hrs) (data not shown). Study of different moisture content of culture on dextranase production by *Bacillus subtilis NRC-B233b* was also investigated. As shown in figure 3, the maximal enzyme production of about 170.6 U/g was achieved in culture of 20 (v/w). The strain showed dextranase production in a broad pH range from 3 to 11. An increase in enzyme production was observed by increasing the alkalinity of the medium and the maximum enzyme production (216.1 U/g) was obtained at pH 10 (Fig 4). Similar results were reported by Yamaguchi and Gocho [27] who showed that *Brevibacterium* dextranase had an optimum dextranase activity at pH 8.0 and 37°C. On contrary, Bhatia et al., [28] 2010 reported that the maximal *Paecilomyces lilacinus* dextranase production was achieved at pH 6.0 and 30°C. The influence of different salts concentrations on dextranase production is shown in fig 5. The results showed that (0.1M) of different salts have variable effects on dextranase production. The enzyme production increased in the presence of KCl and $AlCl_3$ and the maximum enzyme activity was noticed in the presence of $CrCl_3$ (447.58 U/g). The influence of different concentrations of $CrCl_3$ (0.025- 2 M) on the enzyme activity showed that a gradual increase in enzyme activity was obtained by increasing the concentration of $CrCl_3$ from 0 up to 0.175 M. Further increase in salt concentration resulted in a slight decrease in enzyme activity (Fig. 6). This result could be explained that $CrCl_3$ might act as dextranase stimulator. Different organic nitrogen sources were separately added in a concentration of 5g/L to the fermentation media to evaluate their effects on dextranase activity. The results in fig. 7 clearly revealed that, casein, ammonium chloride, yeast extract and corn steep increased the activity. The maximum activity of 1076.8 U/g was obtained in peptone culture. On the other hand, urea and beef extract had adverse effect on dextranase activity. The different concentrations of peptone (1-8 g/L) were added to culture medium to investigate the effect of this important nutrient on enzyme production. The results showed that peptone added in concentration of 20 g/l was the best for dextranse production (data not shown). However, polypeptone was

found to be the most effective nitrogen source for *Brevibacterium* dextranase production as reported by other authors [27]. Non-ionizing radiation, such as ultraviolet (UV) light, exerts its mutagenic effect by exciting electrons in molecules. The goal of this experiment was to improve the activity of the enzyme by using UV mutated variant of *Bacillus subtilis NRC-B233b*. The isolate was exposed to different doses of ultraviolet irradiation. The data in Fig 8 showed that enzyme activity was increased by the time of exposure to UV lamp. Maximum dextranase activity 2842.3 U/g was obtained by using mutant cells exposed to UV for 15 min. Further exposure of culture to UV lamp decreased the dextranase activity gradually. The stability of mutagenic *Bacillus subtilis NRC-B233b* for dextranase production was tested. The results referred to great stability of the mutagenic isolate by using repeated batch technique for 10 times without reduction in its activity. Hild et al., [29] reported in *Paenibacillus* sp. dextranase mutant pool with improved thermostability and activity. The use of ultrafilteration for downstream processing would result in one-step, cost-effective method of recovery for dextranase. Ultrafilteration of the culture broth using membrane PLGC cassett of 10 kD 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 recovery of about 37.3% and 112.2 fold purifications (Table. 1.).

Table1. Partial purification steps of *Bacillus subtilis* NRC-B233b dextranase.

Purification steps	Total activity (U)	Recovered activity (%)	Total protein (mg)	Recovered protein (%)	Specific .activity (U/mg)	Purification fold
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

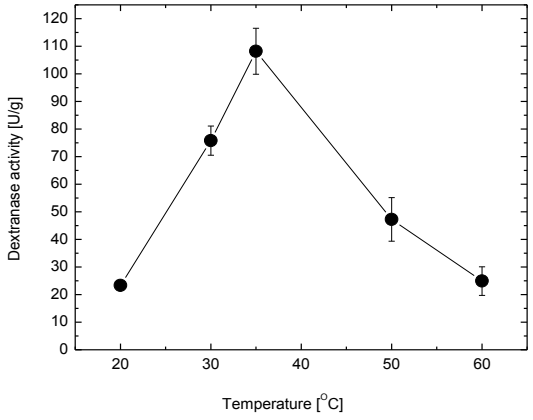

Figure 2. Effect of different temperature on dextranse production from *Bacillus subtilis NRC-B233b*.

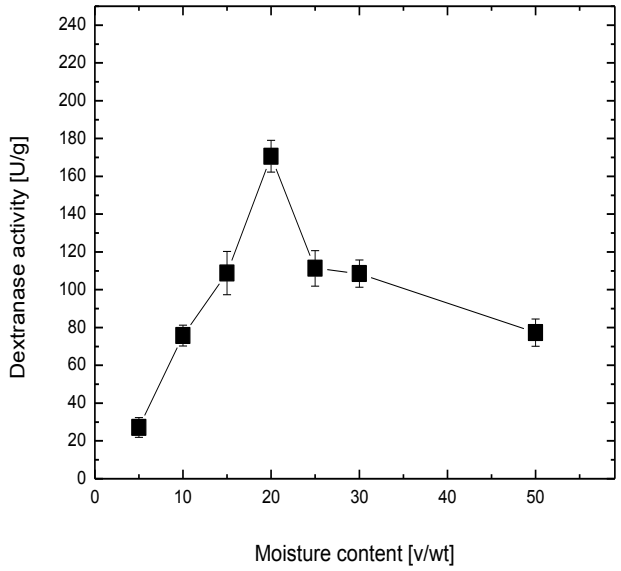

Figure 3. Effect of different moisture content on dextranse production from *Bacillus subtilis NRC-B233b*.

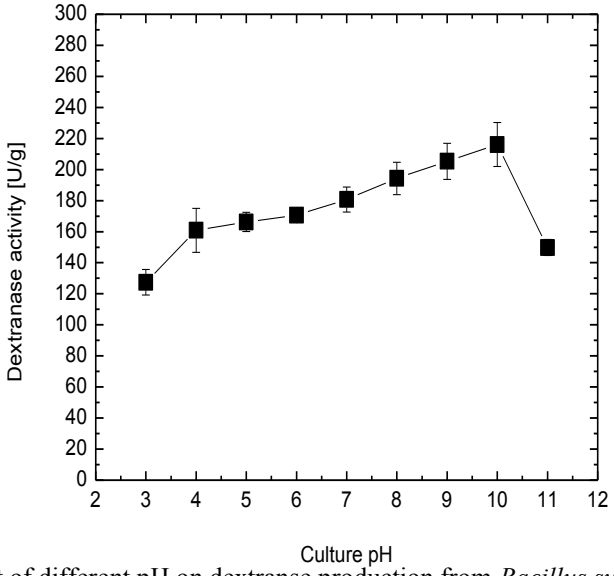

Figure 4. Effect of different pH on dextranse production from *Bacillus subtilis NRC-B233b*.

The properties of the partially purified enzyme in Figs (9,10) clearly appeared 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 [31]. In our study, the optimum pH for dextranase activity was 9. On contrary, Wynter et al., [32] reported that dextranase of *Thermoanaerobacter* sp. exhibited its optimum pH between 5

and 6. Study on 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. On contrary, the alkaline dextranase from a *Brevibacterium* was stable at temperatures below 60°C [27]. 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 [33]. The partially purified dextranase exhibited a broad pH stability range (4–10), especially on the alkaline side (Table. 3). This is in agreement with the recently published work of Kim and Kim [31]. The broad pH stability of our enzyme could be useful in sugar processing, which involves both acidic and alkaline conditions.

Table 2. Thermal stability of *Bacillus subtilis NRC-B233b* dextranase.
Relative values of dextranase activity of enzyme, expressed in %, and obtained by Berridge's method.

Temperature (°C)	**Exposure time (min.)**			
	30	**60**	**90**	**120**
40	100 ± 0.831	100 ± 1.029	100 ± 0.866	100 ± 1.092
45	100 ± 1.016	100 ± 0.917	100 ± 1.029	100 ± 2.008
* **50**	100 ± 2.003	100 ± 0.734	100 ± 2.033	100 ± 1.049
55	100 ± 1.022	100 ± 1.021	100 ± 1.050	100 ±0 .953
60	100 ± 0.820	100 ± 2.019	100 ± 1.070	100 ± 1.030
65	100 ± 0.746	100 ± 0.880	100 ± 2.090	100 ± 1.027
70	100 ± 1.060	100 ± 0.905	100 ± 1.037	100 ± 1.025
75	100 ± 0.909	100 ± 1.034	80.6 ± 0.930	80.6 ± 2.057

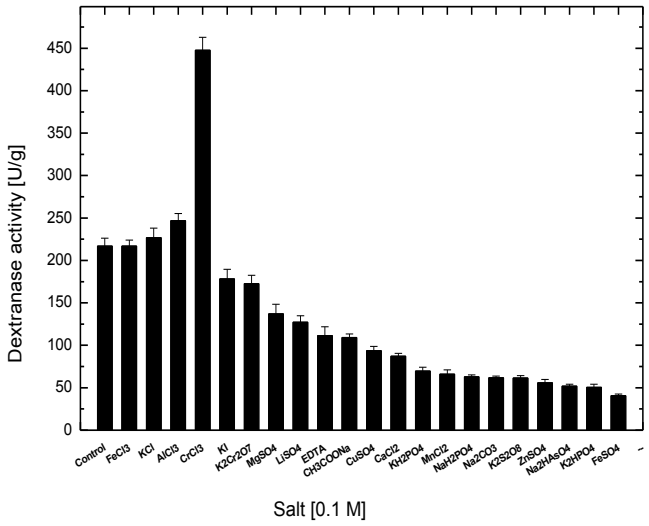

Figure 5. Effect of different salts on dextranase production from *Bacillus subtilis NRC-B233b*.

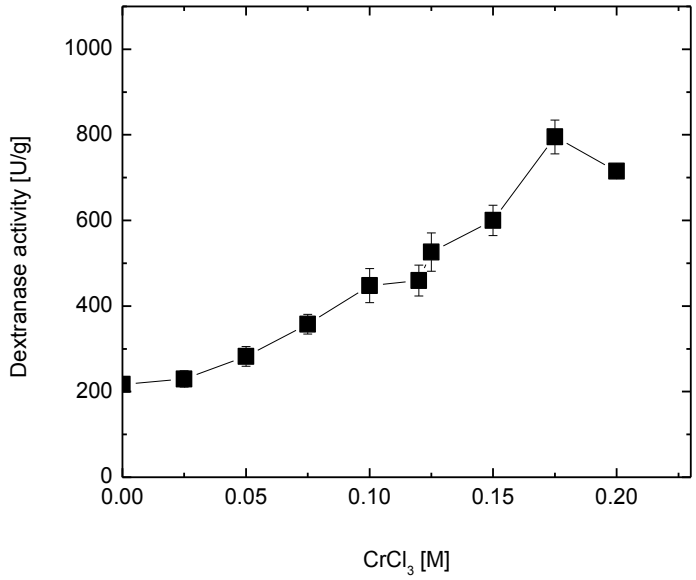

Figure 6. Effect of different concentrations of Chromium chloride on dextranase production from *Bacillus subtilis NRC-B233b*.

The partially purified dextranase was greatly affected by the metal ions addition, the halophilic feature of this enzyme appeared clearly when the enzyme activity increased about 4 fold in the presence of 1.92 M NaCl (Fig.11). 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

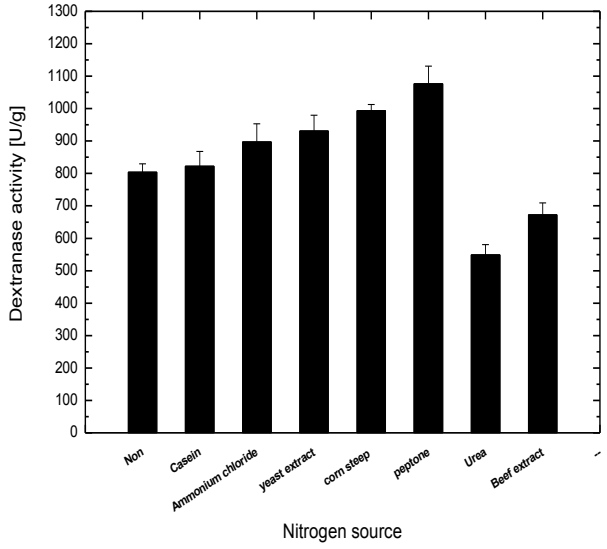

Figure 7. Effect of different nitrogen sources on dextranse production from *Bacillus subtilis NRC-B233b*.

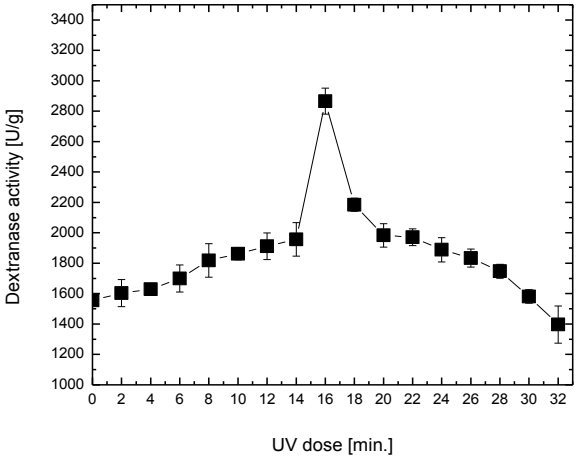

Figure 8. Effect of UV irradiation (min.) on the activity of dextranase.

the protein molecules [30]. It was suggested that the halophilic enzymes has 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 effect of different salts showed that EDTA, $CaCl_2$, KCl , Mg_2SO_4 and $CrCl_3$ all enhanced enzyme activity and the relatives activities (%) were 264.4 ±0.053, 228.8 ±0.22, 207.6 ±0.015, , 181.25 ±0.029 and 125 ±0.031 respectively (data not shown). Whereas dextranase activity was strongly inhibited by $MnCl_2$ and $AlCl_3$, and the retained activities were 31.3±0.001, 36.9±0.001, respectively. On the other hand

$CuSO_4$ and $ZnSO_4$ reported 77.5±0.064 and 77.5±0.064 retained activity. Sugiura et al., [34] 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. On contrary *Thermotoga lettingae* TMO dextranase activity was not significantly affected by the presence of metal ions, except for the strong inhibition by 1 m M Fe^{2+} and Ag^{2+} [31]. The substrate specificity of *Bacillus subtilis NRC-B233b* partially purified dextranase on different types of carbohydrates and the rate of enzyme hydrolysis with various glycosidic linkages was done. This enzyme could act efficiently on high and low molecular weight dextran. Where it could preferentially split a series of dextrans and their derivatives (Sephadex), the rate of hydrolysis was dependent on the molecular weight of the substrate. The results presented in

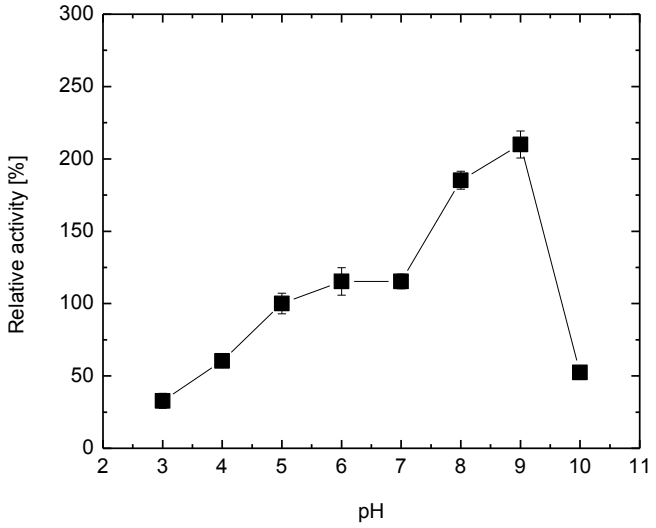

Figure 9. Effect of different pH on partial purified dextranse activity.

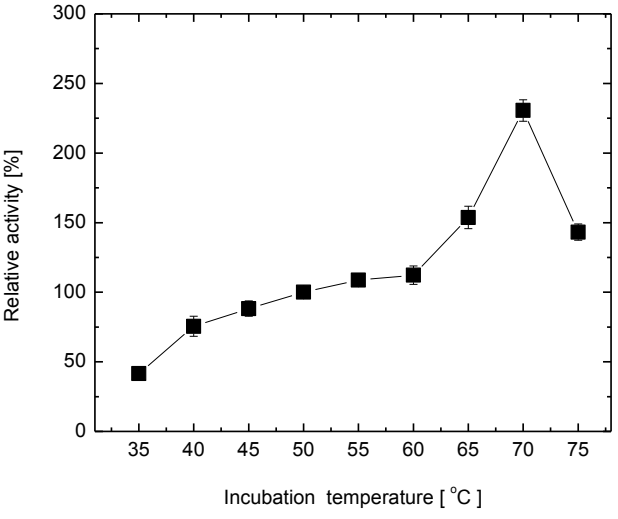

Figure 10. Effect of different temperatures on partial purified dextranse activity.

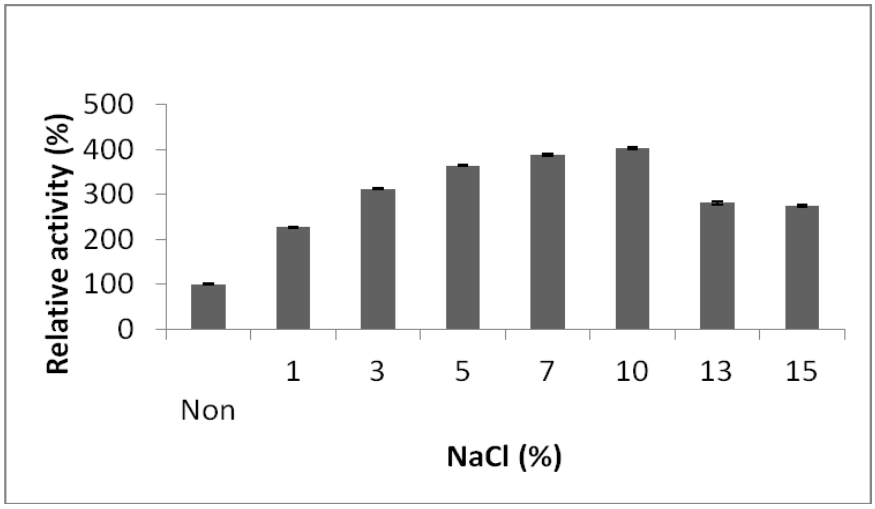

Figure 11. Effect of different NaCl concentrations on partial purified dextranse.

Table3. pH stability of the partially purified *Bacillus subtilis NRC-B233b* dextranase. Relative values of dextranase activity of enzyme, expressed in %, and obtained by Berridge's method.

pHs	**Exposure time (min.)**			
	30	**60**	**90**	**120**
5.0 *	100 ± 0.331	100 ± 0.844	90 ± 1..066	83 ± 0.992
6.0	100 ± 0.0216	100 ± 0.217	100 ± 0.829	100 ± 0.708
7.0	100 ± 0.303	100 ± 0.534	100 ± 0.933	100 ± 1.049
8.0	100 ± 0.666	100 ± 1.021	100 ± 0.650	100 ± 0.553
9.0	100 ± 0.546	100 ± 0.919	100 ± 0.66	100 ± 0.930
10.0	100 ± 1.046	95 ± 0.180	81 ± 0.590	80 ± 0.627

Table 4. reported that blue dextran (1 kD) gave the maximum relative activity (273.8%). It was also reported that *Penicillium notatum* dextranase could act on different types of dextran and the rate of hydrolysis was independent of the molecular weight of substrate [35]. The digestion products from the Sephadex derivatives showed the presence of reducing sugars. The previous result suggests that dextranase is an interesting enzyme for removal of dental cariogenic plaque deposits [36]. A great dextranolytic activity was found when starch (α-1, 4 and α-1, 6 glycosidic linkages), amylopectin, inulin and sucrose (α-1, 2 glycosidic linkages) were used as substrates.

Table 4. Action of *Bacillus subtilis NRC-B233b* dextranase on various carbohydrates.

Substrates	Main linkage	Relative activity (%)
*Dextran (250kD)	α-1,6	100
Dextran (200-275 kD)	α-1,6	79.2
Blue dextran (2 kD)	α-1,6	273.8
Sephadex G-100 (1-10 kD)	α-1,6	112
Starch	α-1,6	228
Amylopectin	α-1,6	165
Maltose	α-1,4	71
Inulin	α-1,4	170
Sucrose	α-1,2	100
*Control.		

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 [37-39]. Degradation of alpha-linked D-gluco-oligosaccharides and dextrans by an isomalto-dextranase preparation from *Arthrobacter globiformis* T6 was also reported by other authors [39]. In laboratory study, the treatment of viscous sugar cane juice with different dextranase concentrations resulted in clear visual dextran hydrolysis. The results showed gradual decrease in viscosity 68%, 72%, 96% respectively (Table. 5). Sugarcane juices have high levels of glucose, fructose, and particularly sucrose. Mannitol will also often be present when dextran is present in cane juice because both are formed mainly from *Leuconostoc mesenteroides* [40]. These short and long chain carbohydrate sugars could potentially affect the activity of dextranase if applied in sugar factories. None of the sugars tested showed any adverse effect on dextranase activity. This result confirms the capability of application of *Bacillus subtilis NRC-B233b* dextranase in sugar industries. Finally, the crude enzyme was highly tolerant to repeated freezing and thawing, the activity remaining at 100% for three months.

Table 5. The effect of different *Bacillus subtilis NRC-B233b* dextranase concentrations on sugar cane juice viscosity.

Viscosity Reduction (%)	Enzyme added (U)
68	237
82	475
96	712
97	950
97	1185

Conclusion

In this study we focused on isolation of halophilic bacteria from honey as new source. The mutagenic honey isolate produced a novel halophilic constitutive dextranase characterized

by unique features, like halophilic, thermostability and pH stability. Furthermore, 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. The *Bacillus subtilis NRC-B233b* dextranase showed wide substrate specificity, also it not affected by the presence of some sugars. The laboratory experiment highly proved that this enzyme could play role in sugar cane industry.

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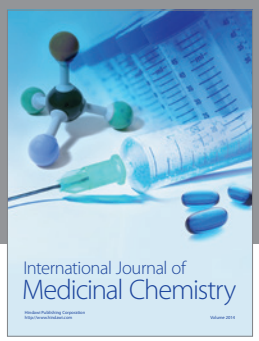

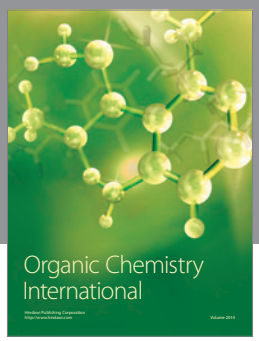

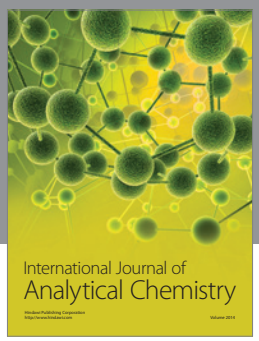

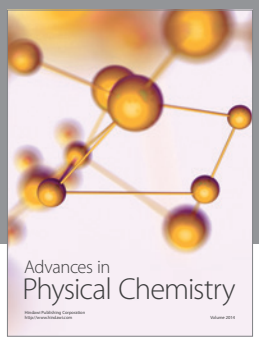

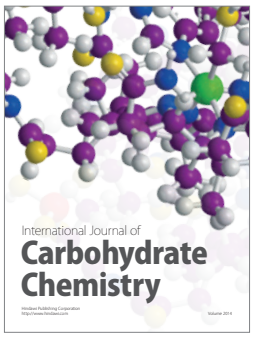

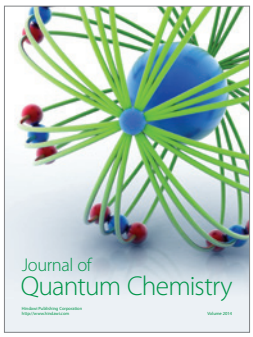

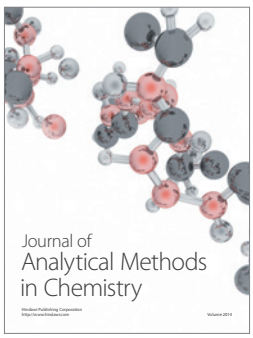

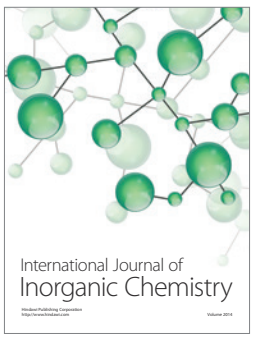

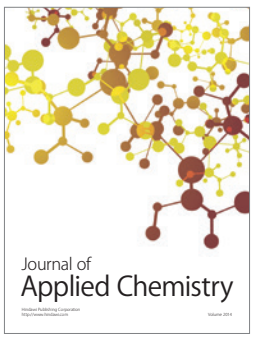

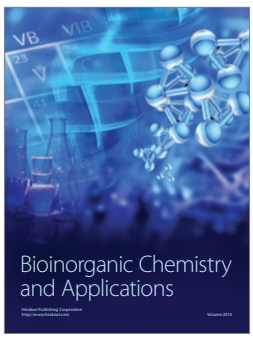

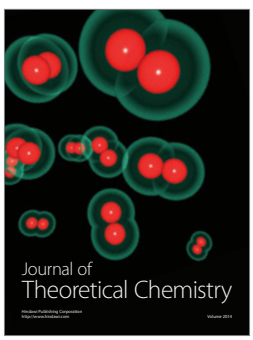

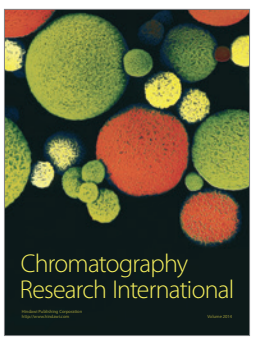

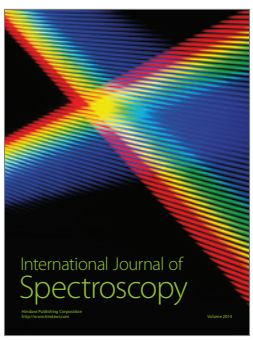

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