Production of dextranase by *aspergillus fumigatus* NRC-F103 and its application in cane juice treatment and enhancing ethanol production from sugarcane molasses

Mohamed Fadel, Fatima H. Motawe, Amira M. Roshdy

Department of Microbial Chemistry, Genetic Engineering and Biotechnology Research Division, National Research Centre, Dokki, Giza, Egypt

Correspondence to Amira M. Roshdy, PhD, Department of Microbial Chemistry, Genetic Engineering and Biotechnology Research Division, National Research Centre, Dokki, Giza 12311, Egypt. Tel: +20 122 535 2581; fax: +20 3337 0931; e-mail: amiraroshdy82@yahoo.com

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Background and objectives
One of the important applications of dextranase enzyme is preventing dextran accumulation in sugarcane juice as well as, consequently, for enhancing the resultant fermentable reducing sugars and ethanol yield in the fermentation of sugarcane molasses by yeasts.
Materials and methods
Different materials and methods were used for fungal strains [screening, mutation ultraviolet (UV), inoculum preparation, cultivation type 'solid-state fermentation,', culture substrates], dextranase (production, assay, soluble protein determination), purification, characterization, application in ethanol production by fermentation from sugarcane molasses, dextran estimation, and determination of reducing sugars.
Results and conclusion
Six fungal strains (namely *Aspergillus oryzae* FK-923, *Aspergillus niger* F-93, *A. niger* F-258, *Aspergillus awamori* NRC-F18, *Aspergillus fumigatus* NRC-F103, and *Trichoderma viride* NRC-F107) were screened on sorghum, sugar beet pulp, wheat bran, and orange peels using the solid-state fermentation technique to produce dextranase enzyme. The fungus *A. fumigatus* NRC-F103 cultivated on orange peels showed promising enzyme yield than other tested fungal strains. Then, optimization of culture conditions for dextranase production was carried out. Moisture content, initial pH value, incubation temperature, and incubation period were optimized to be 2 : 1 (v/w), 5.0, 35°C and 96 h, respectively. Five inorganic nitrogen sources were trailed at equivalent levels as sole nitrogen in the fermentation medium did not result in any increase in enzyme activity. Subjecting the fungal to UV resulted in a 75% increase in enzyme activity corresponding to the mother strain before subjecting to UV. Under the above conditions, 118 U/g original substrate was obtained. Isopropanol 1 : 1 (v/v) was applied for precipitation enzyme protein, as 32% of total protein involving 68% of total enzyme activity was obtained and specific activity was 42.94 U/mg protein compared with 16.4 U/mg protein in the culture supernatant. A study on obtained dextranase showed that it has an optimum that pH ranged from 4.5 to 5.5 as well as it gave the highest activity when incubated between 35 and 40°C. Promising results were obtained when the enzyme was applied in cane juice to prevent accumulation of dextran. Enzyme supplementation to diluted sugarcane molasses (26%, w/v) resulted in an increase in reducing sugars by 2.64%. Ethanol was increased by about 2.36% (v/v) in the fermentation medium supplemented with an enzyme compared with the unsupplemented medium and the fermentation efficiency increased from 89 to 92.5%.

Keywords:
*A*spergillus *fumigatus* NRC-F103, dextranase, ethanol production, fermentation, fungi, orange peels, sugarcane juice, sugarcane molasses

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Introduction

Dextranase is an enzyme, which hydrolyzes the α-1,6-glucosidic linkages in dextran. The enzyme has several applications in medicine and sugar industry. The presence of dextran in sugarcane mills has many negative effects that lead to sucrose loss, increased viscosity of processed syrups, and results in less sucrose crystallization recovery efficiency [1,2] as well as the presence of dextran leads to deposits of insoluble polymers on the processing equipment [3–7]. Dextranase can be supplemented in dental toothpaste as expellant whitening agent in the treatment of dental plaque. So, dextranase has been used in toothpastes involved as a component additive for dental care, since it destroyed dextran, which is responsible for dental

plaque. Dextranase can prevent or inhibit the synthesis of glucans deposit on teeth. The enzyme is applied for mashing low molecular weight dextran and cytotoxic dextran conjugate and dextranase as an enhancer of antibiotic activity in endocarditis [8,9]. Fungal dextranase, 1,6-D-glucan 6-glucanohydrolase (EC 3.2.1.11), is an enzyme, which catalyzes endohydrolysis of –(1,6)-D-glycoside linkages in random sites of dextran. Isomaltose, isomaltotriose, and a small amount of D-glucose, jointly with traces of higher oligomers, are the major reaction products. However, a variation in reaction products and substrate specificities of dextranases from diverse sources is obvious. For instance, endo-dextranase from a *Penicillium* sp. degrades cyclodextrins into glucose and isomaltose. All fungal dextranases (EC 3.2.1.11) can degrade the cross-linked dextran Sephadex. Fungi are the widespread source for the extracellular endodextranases (EC 3.2.1.11) and exhibit a higher enzyme activity than dextranases from yeasts and bacteria. There is only a single report on intracellular fungal dextranases found in *Penicillium funiculosum* NRRL-1132 and *Penicillium lilacinum* NRRL 896. The hydrolysis products of *Penicillium notatum* dextranase are isomaltose and isomaltotriose with a little amount of glucose, as in most mold dextranases. Fermentable sugars are released from dextran much faster and in better quantities by accidental attack of endodextranases in comparison with terminal end-group attack of exoenzymes. Therefore, 67–74% conversion of dextran into sugar syrup is accomplished by *P. notatum* dextranase [9].

Moreover, dextranase has a vital role in directed synthesis of isomaltooligosaccharides, which exhibit prebiotic effects since its involvement in toothpaste prevent the colonization of streptococci that causes destruction of the calcified tissue [10]. Dextranase is considered to be usable in digestion methods of dextran polymer to obtain isomaltooligosaccharides [11]. Various fungi were screened to find the best dextranase producer and the culture conditions for dextranase production from *Penicillium lilacinus*, *P. notatum* [12], *Paecilomyces* lilacinus [13], *P. funiculosum*, and *Penicillium aculeatum* [14], some bacteria such as *Bacillus subtilis*, *Leuconostoc dextranicus*, and *Lipomyces*, and yeast strains as *Saccharomyces cerevisiae* [14].

Materials and methods

Microorganisms

Nonmycotoxins-producing six fungal strains, namely *Aspergillus oryzae* FK-923, *Aspergillus niger* F-258, *Aspergillus awamori* NRC-F18, *Aspergillus fumigatus* NRC-F103, and *Trichoderma viride* NRC-F107 were obtained from Microbial Chemistry Lab., National Research Center, Dokki, Cairo, Egypt, and maintained on potato dextrose agar (PDA) slants at 30°C for 72 h.

Inoculum preparation

Inoculum was made from 3-day-old PDA cultures. The inoculum (containing 10^6–10^7 spores) was suspended in 0.05 M acetate buffer. The suspension, when necessary, was diluted with sterilized 0.05 M acetate buffer (pH 5.0) to give a spore count within the predetermined range. One milliliter of inoculum was added to each, cooled, and sterilized (autoclaving at 121°C for 15 min) 5 g of 60% v/w moisture substrate in 250 ml capacity conical flasks.

Solid-state fermentation and enzyme production by different fungi cultivated on various substrates

Screening of fungal strains for dextranase production was studied in 250 ml Erlenmeyer flasks containing 5 g of wheat bran moistened to 60% (v/w, ml/g) with distilled water. A measure of 1 ml of spore suspension (10^6 spores) from each fungal was used as inoculum. The cultures were incubated at 30°C for 4 days. At the end of the incubation period, 100 ml of distilled water was added to each flask, blended with shaking at 150 rpm for 30 min, and then harvested by filtration. The filtrates were saved as sources of crude enzyme. The selected fungal strain and substrate were incubated for 192 h at 30°C and the culture was taken at 24 h intervals to detect the optimum incubation period. Orange peels were moistened with distilled water to a moisture level of 1 : 2 (v/w) under the optimum incubation period to determine the more suitable moisture content for enzyme production. Acetate buffer (0.1 M) was used for adjusting the initial pH of the fermentation medium to different values from 3.5 to 7.5 to study the effect of initial pH on enzyme secretion. The fungus was incubated under different temperatures, that is, 24, 27, 30, 33, and 36°C to study the effect of temperature on enzyme production. Nitrogen sources, that is, urea, diammonium phosphate, ammonium sulfate, sodium nitrate, and potassium nitrate at a level of 0.92 mg N/g solid substrate were applied in the fermentation medium to study their effect on enzyme yield.

Effect of ultraviolet irradiation of selected microorganism on its dextranase production yield

Mutagenesis of the wild type by ultraviolet (UV) irradiation was carried out according to the method

described by Abdel-Aziz *et al.* [15] as follows: dilutions of mother fungal isolate suspension obtained from fungal culture on PDA slants after 5 days incubation at 30°C were crushed in 10 ml sterilized water, introduced in 100 ml capacity conical flask, and then diluted to be 10^{6-7} spores/ml. Later, they were distributed into 9 cm diameter sterilized Petri dishes occupied by 4 ml from the above culture spores. These were exposed to UV radiation for different times ranging from 20 to 120 min in a UV chamber (70×50 cm) keeping the distance of the UV source at 15 cm. After UV radiation, they were kept in dark for stabilization. Parent type and UV-treated fungal spore suspensions of 0.1 ml was inoculated in a 5 ml diameter Petri plate containing PDA medium and incubated at 30°C. After 72 h, the fungal growth was crushed in 10 ml sterilized water and used as inoculum.

Enzyme assay

The dextranase activity was assayed by estimating the amount of reducing sugars released under assay conditions. Activity was measured by determining the amount of reducing groups released according to the (3, 5 dinitro salicylic acid) DNS method described by Miller [16]. The substrate used for the assay was 1% dextran in 0.1 M acetate buffer pH 5.0. The assay mixture was prepared with the following components: 0.1 ml enzyme, 0.9 ml of 1% solution of dextran. Blank was prepared for each sample by boiling the reaction mixture before the addition of the substrate. Tubes were incubated at 50°C for 30 min. The devolved color was a measure of spectroophotometry at 540 nm. The amount of reducing sugars released per ml per minute was calculated from the standard curve of dextrose (0.1–1.0 mg /ml). One unit of enzyme activity is defined as the enzyme that releases 1 µmol/ml/min dextrose under standard assay conditions.

Determination of reducing sugars in molasses

Reducing sugars in sugarcane molasses was determined by the DNS method reported by Miller [16].

Protein determination

Enzyme protein was determined by the Folin-phenol method described by Lowry *et al.* [17].

Partial purification of enzyme

The filtrate eluted from the fermented substrate was centrifuged at 6000 rpm for 20 min under refrigerated condition. A measure of 1 : 1 cold isopropanol was added slowly to the culture supernatant. The precipitated protein was separated by centrifugation at 6000 rpm for 30 min at 4°C. The precipitated protein was dissolved in sodium acetate buffer (0.1 M pH 5). Protein concentration, enzyme recovery, enzyme activity, and specific activity were determined.

Characterization of dextranase

The optimum temperature of dextranase was detected by incubating the enzyme at different temperatures from 35 to 80°C in assay buffer and then measuring the activity by standard assay. The more suitable pH for enzyme was evaluated by incubating enzyme at varying pH values of the reaction mixture between 3.0 and 8.0 at an increment of 0.5 using citrate and phosphate buffers. Activity was then assayed.

Estimation of dextran

Estimation of dextran in cane juice and molasses was carried out according to Analysis of the Association of Official Analytical Chemists (AOAC) [18].

Application of dextranase to prevent dextran formation in cane juice

Different concentrations (2, 4, 6, 8, and 10 U) of extracted crude dextranase were added to 100 ml sugarcane juice and the formed dextran content in juice was estimated at different time intervals (1, 3, 5, 8, 18, and 24 h).

Application of enzyme in ethanol production by fermentation from sugarcane molasses

Partially purified dextranase obtained by isopropanol (1 : 1) was applied at different enzyme units/100 g molasses according to the methods reported by Fadel *et al.* [19]. The performance of reducing sugars was determined by the DNS method of Miller [16].

Molasses fermentation for ethanol production

S. cerevisiae F-307 was used to ferment sugarcane molasses before and after enzyme addition. Fermentation efficiency was calculated according to Fadel [20].

Results and discussion

Dextranase activity obtained from six fungal strains cultivated on wheat bran under the solid-state fermentation method for dextranase production

Dextranase activities obtained from nonmycotoxins-producing six fungal strains namely *A. oryzae* FK-923, *A. niger* F-93, *A. niger* F-258, *A. awamori* NRC-F18, *A. fumigatus* NRC-F103, and *T. viride* NRC-107 cultivated on wheat bran are illustrated in Fig. 1. The figure shows that *A. fumigatus* NRC-F103 was a more promising fungus to test enzyme production

(16.8 U/g original substrate), followed by *A. niger* F-93. Various fungi were screened to find the best dextranase producer and the culture conditions for dextranase production from *P. lilacinus* and *P. notatum* [12] and *P. lilacinus* [13].

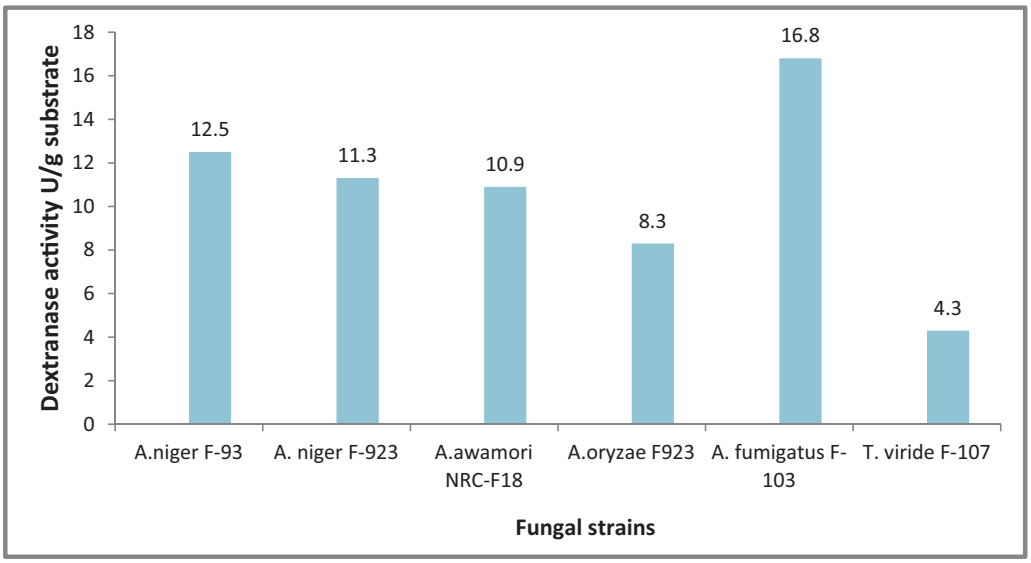
Effect of cultivation substrate on dextranase production by *Aspergillus fumigatus* NRC-F103 to select the most promising substrate for enzyme production

The dextranase activity obtained from cultivated *A. fumigatus* NRC-F103 on wheat bran, maize flour, sorghum flour, sugar beet pulp, and orange peels is illustrated in Fig. 2. The fungus showed more enzyme secretion when cultivated on orange peels (45.9 U/g original substrate), followed by sugar beet pulp (38.5 U/g original substrate) with the advantage for orange peels. The obtained data results can be discussed in light of the difference in chemical composition; configuration of the molecules of the substrate permit more oxygen available for fungus and mycelium penetration through the substrate.

Effect of different incubation temperatures on dextranase production by *A. fumigatus* NRC-F103 cultivated on orange peels

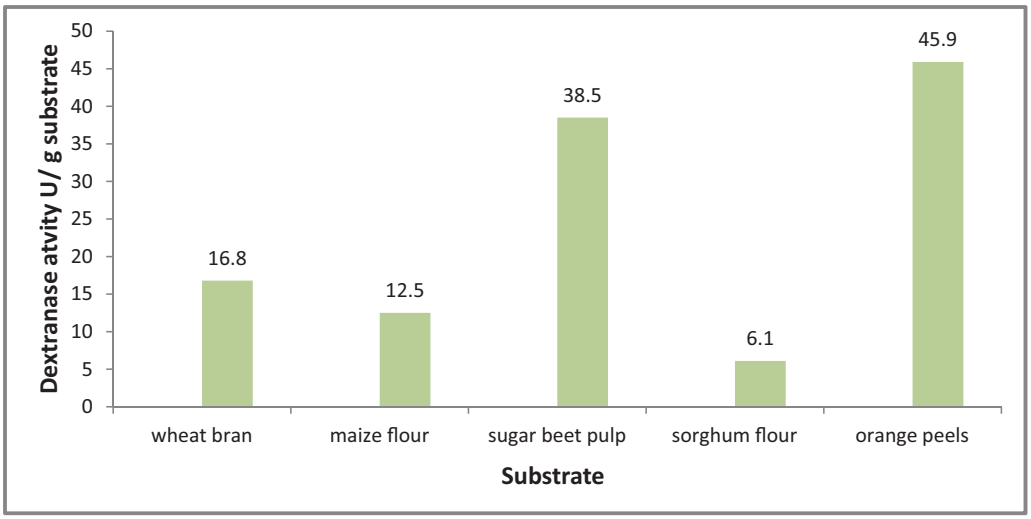
The effect of incubation period on dextranase production by *A. fumigatus* NRC-F103 cultivated on orange peels is illustrated in Fig. 3. The presented data

Figure 1



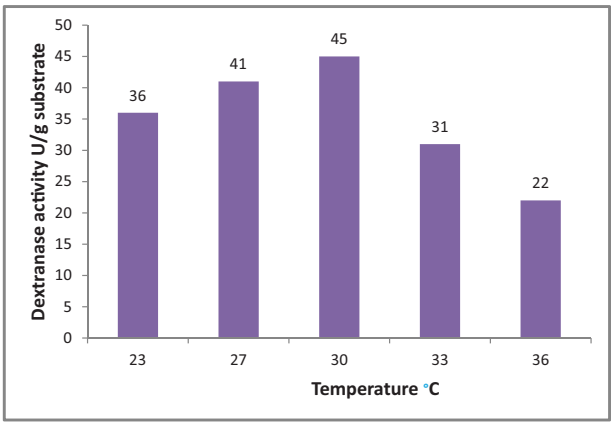
Dextranase activity obtained from five fungal strains cultivated on wheat bran under solid-state fermentation method for dextranase production.

Figure 2



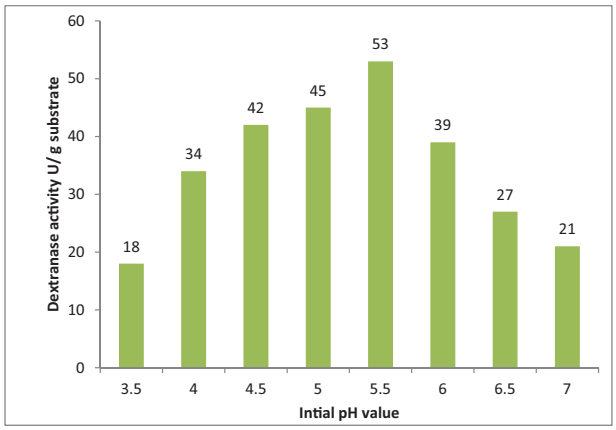
Effect of cultivation substrate on dextranase production by *Aspergillus fumigatus* NRC-F103.

Figure 3



Effect of different incubation temperatures on dextranase production by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels.

Figure 4



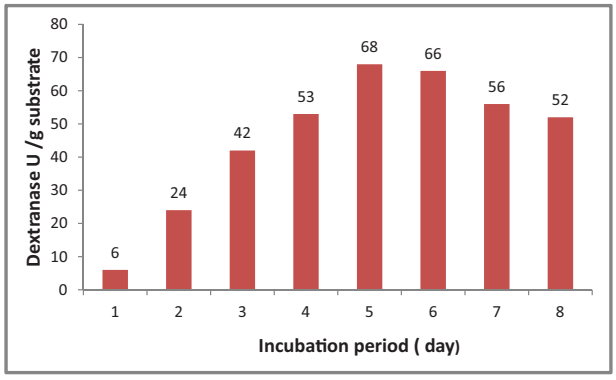
Effect of initial pH value of fermentation medium on dextranase production by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels under solid-state fermentation at 30°C after 96 h.

plotted in Fig. 3 illustrates that incubation temperatures (23–36°C) affect greatly the enzyme production produced by *A. fumigatus* NRC-F103. The optimum temperature was 30°C as the activity of dextranase reached 45 IU/g original substrate. The enzyme production affected negatively in high incubation temperatures than low incubation temperatures. The result can be discussed in light of slow growth of fungus at low temperatures and thermal inactivation of the enzyme at high temperatures. The obtained results agree with that reported for many fungus such as *Lipomyces starkeyi* [21] and *P. funiculosum* [15]. Maximum dextranase production at 30°C was also reported by Koenig and Day [21] as well as *P. lilacinus* [13]. Also, Erhardt *et al.* [22] reported a temperature of 26°C for optimum production of dextranase from *P. aculeatum*.

Effect of initial pH value of fermentation medium on dextranase production

As to the effect of initial pH value of fermentation medium on dextranase production, the data presented in Fig. 4 indicates that the pH of the growth medium has an important effect on the enzyme released into the fermentation medium. The enzyme increased by increasing the pH value from 3.5 to 5.5 and then decreased when the initial pH was above 5.5. The maximum enzyme activity registered at pH 5.5 was 53 IU/g original substrate. Previous research has stated the same pH for fungal dextranase production by several fungi strains. pH 5.5 was chosen as more suitable for dextranase production from *P. notatum* [23], pH 5 as the optimum for dextranase production by *Aspergillus sinusitis* [24]. Cheng *et al.* [25] and Bhatia *et al.* [13] stated that the optimum pH for dextranase production is 6.0 by *P. funiculosum* and *P. lilacinus*, respectively.

Figure 5

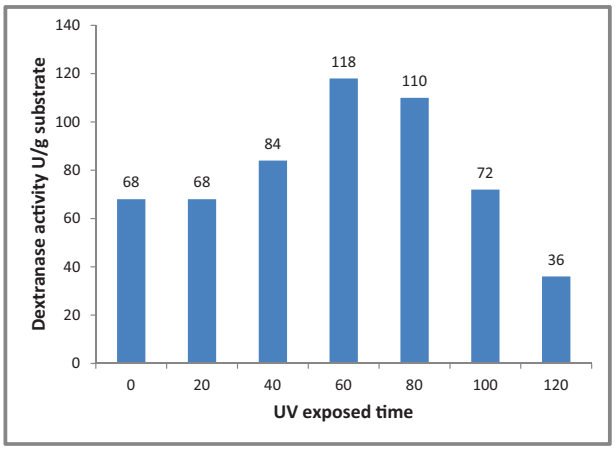


Effect of the incubation period on the production of dextranase by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels under solid-state fermentation initial pH of 5.5 and at 30°C temperature.

Effect of incubation period on the production of dextranase

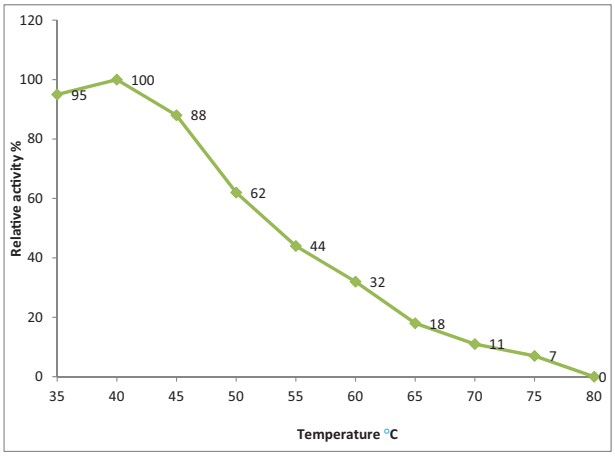
The period required for incubation depends on the growth rate of microorganism on the substrate and on enzyme production pattern. Figure 5 shows that the fungus enzyme began released dextranase into the fermentation medium after 1 day. The enzyme increased by increasing the fermentation period and reached its maximum after 5 days and after that decreased gradually. The maximum enzyme activity achieved was 68 IU/g of the original substrate. Previous researches stated the incubation time for fungal dextranase production by many fungal strains cultivated on agricultural wastes under solid-state fermentation. Arnold *et al.* [26] also reported maximum production of dextranase after 5 days of incubation by *Sporothrix schenckii*. However, Shimizu *et al.* [27] reported that the production of dextranase by *Fusarium* spp. was after 7 days of incubation at 30°C.

Figure 6



Effect of UV subjected time on the production of dextranase by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels after 5 days under solid-state fermentation initial 5.5 pH and at 30oC temperature. UV, ultraviolet.

Figure 7

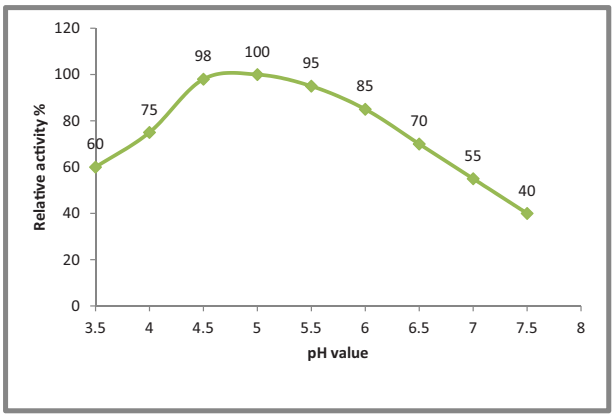


Effect of incubation temperature on the dextranase activity produced by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels after 5 days under solid-state fermentation initial 5.5 pH and at 30°C temperature.

Effect of ultraviolet irradiation of the selected mutant fungus on its dextranase production yield

The effect of UV irradiation of the selected mutant fungus on its dextranase production yield is illustrated in Fig. 6. The figure indicated that the exposure of fungal spores to UV for 1 h led to 75% increase in enzyme activity compared with the mother strain before subjecting to UV. Enzyme released into the fermentation medium after five reached 118 IU/g of the original substrate instead of 68 IU/g original substrate for mother isolate. Mutagenesis using UV was studied for the enhancement of dextranase production from different fungal strains by Abdel-Aziz *et al.* [15] could increase the dextranase production by 189% from *P. funiculosum*.

Figure 8



Effect of pH on the dextranase activity produced by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels after 5 days under solid-state fermentation initial 5.5 pH and at 30°C temperature.

Characterization of dextranase

Temperature

The temperature characterization of dextranase enzyme is illustrated in Fig. 7. The figure illustrates that the obtained enzyme gave its highest activity between 35 and 40°C and decreased sharply by increasing the temperature till it became completely active at 80°C. The obtained data are in agreement with that reported by Elvira *et al.* [28] and Madhu *et al.* [29].

Optimum pH

The pH characterization of dextranase enzyme, is illustrated in Fig. 8. The figure illustrates that the obtained enzyme gave its highest activity between pH at a range of 4.5–5.5. A decrease in activity was seen after or before the previous range, whereas Shamsolahrar *et al.* [30] reported that the optimum pH of *P. lilacinum* dextranase was 4.5. On the other hand, Hattori and Ishibashi [31] reported that the dextranase from *Chaetomium gracile* was stable in the most wide range of pH value (pH 5–10).

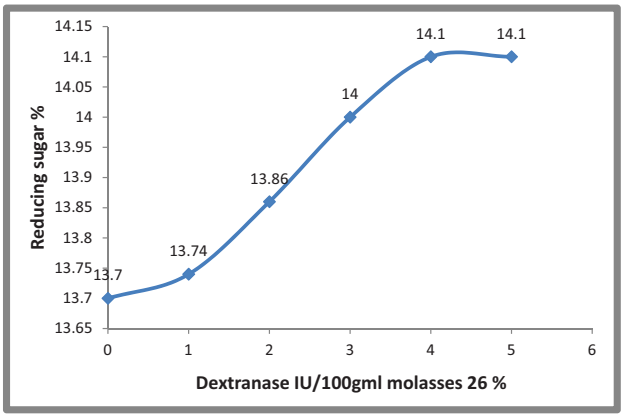
Application of dextranase to prevent dextran accumulation in sugarcane juice

In sugar production, dextrans are undesirable compounds synthesized by contaminant microorganisms from sucrose, increasing the viscosity of the flow and reducing industrial recovery, bringing about significant losses [32]. The use of the dextranase enzyme is the most efficient method for hydrolyzing dextrans at sugar mills. Table 1 shows the effect of supplementation of dextranase with sugarcane juice at deferent levels for 24 h. Data clears the positive effect and the advantage of application of dextranase in preventing the accumulation of dextran in sugarcane juice and this means that the addition of enzyme meets the demands

Table 1 Prevention of dextran accumulation in sugarcane juice by adding dextranase produced by *Aspergillus fumigatus* NRC-F103 cultivated on orange peels under solid-state fermentation under 66% moisture at an initial pH of 5.5 for 5 days at 30°C

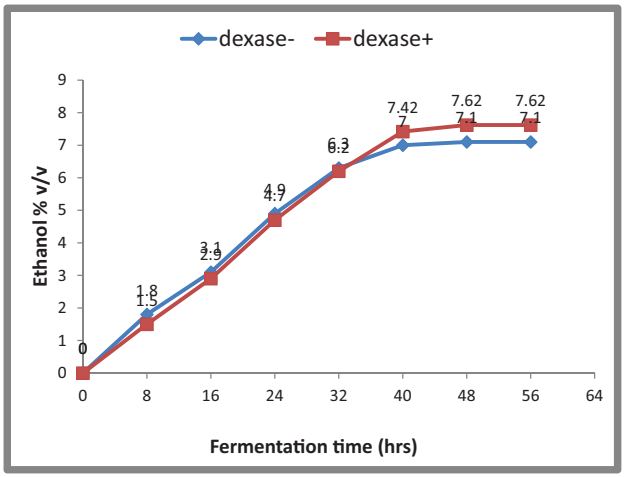
Dextranase units (/100 ml cane juice)	Dextran (mg/100 ml cane juice)					
	Time (h)					
	0	3	5	8	18	24
0	2.28	3.05	5.11	11.32	18.24	26.60
1	2.28	3.00	7.50	11.80	15.00	22.16
2	2.28	2.55	6.12	9.20	14.64	18.60
3	2.28	2.24	3.88	6.42	8.64	10.98
4	2.28	2.16	3.20	4.86	6.12	7.44
5	2.28	2.08	2.29	4.56	5.08	6.22

Figure 9



Effect of dextranase addition on fermentable sugars content in sugar cane in diluted sugar cane molasses.

Figure 10



Effect of fermentation time on ethanol production.

of the sugar industry, as the prevention of dextran accumulation decreased the viscosity of the juice and increased the obtained sugar yield, consequently increasing the efficiency of the industrial process. The addition of dextranase to cane juice in sugar manufacturing overcome the problems caused, the loss of sucrose, increase in viscosity of processed syrups, and poor recovery of sucrose due to inhibition of crystallization [33,34]. Suitable addition of enzyme to the juice is demanded and this is in agreement with the previous findings of Ellis and Miller [35] as they showed that the treatment with these enzymes in the syrup is needed to increase the dose. Many investigations suggested a minimization of dextran levels in the sugar factory by different methods even with the chemical additions. More recent approaches propose the application of dextranase for the removal of performed dextran. The enzymatic method is quick, easy, and can be practiced at a commercial scale [33,34].Application of dextranase to increase fermentable sugars in diluted sugarcane molasses

Data presented in Fig. 9 illustrate the effect of dextranase as a hydrolyzing enzyme on reducing the sugar content of diluted sugarcane molasses. When dextranase was added at 4.0 IU/100 ml to the molasses medium, there was an increase in fermentable sugars according to the action of dextranase hydrolyzing from 13.7 to 14.1% (w/v) . The performance means that the fermentable sugars increased by about 2.6%. Previous researchers reported the positive effect of supplement dextranase for hydrolyzing dextran at low Brix sugarcane molasses [34–37].

Application of dextranase in ethanolic fermentation of sugarcane molasses

Figure 10 illustrates the comparison between the ethanol yield from sugarcane molasses before and after dextranase addition. It shows that the ethanol yield after complete fermentation was 7.1% without the addition and increased to 7.6% after dextranase addition. The increase in ethanol yield from hydrolyzing dextran in molasses to glucose led to increasing fermented sugar in the fermentation medium and is consequently fermented by *S. cerevisiae* to ethanol. Thus, selection of yeast strain as well as addition of dextranase are necessary for ethanol yield production and consequently fermentation efficiency [19,20].

Conclusion

Dextranase enzyme can be produced economically by culturing a mutant of fungus *A. fumigatus* NRC-F103 on agro-industrial waste orange peels under solid-state fermentation. Promising results were obtained when the produced enzyme was applied to the cane juice to prevent the accumulation of dextran. Enzyme supplementation to diluted sugarcane molasses (26%, w/v) resulted in an increase in reducing sugars by 2.64%. Ethanol was increased by about 2.36% v/v in the fermentation medium supplemented with enzyme compared with the unsupplemented medium and the fermentation efficiency raised from 89 to 92.5%.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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