Production and immobilization of invertase from *Penicillium* sp. using orange peel waste as substrate

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Background and objective
Invertases represent a group of industrially important enzymes that catalyze the breakdown of sucrose into fructose and glucose. The objective of the present work was the production of invertase enzyme from *Penicillium* spp. using orange peel waste as substrate under optimum conditions, and also immobilization and characterization of invertase using chitin as carrier.
Materials and methods
The fungal strain *Penicillium* spp. and *Trichoderma viride* were studied for invertase production using different agricultural wastes as substrate. Some parameters such as concentrations of substrate, incubation time, and inoculum size affecting invertase produced by *Penicillium* spp. using orange peel as substrate were investigated. The invertase produced by *Penicillium* spp. was partially purified with acetone (60%). Different carriers such as chitin, foam, and saw dust were used for invertase immobilization by covalent binding. The characterizations of immobilized invertase on chitin such as substrate concentrations, pH values, temperature, time of the reaction, thermal stability, and some metal ions were determined.
Results and conclusion
On comparison between production of invertase from *Penicillium* spp. and *T. viride* using different agricultural wastes, it was found that *Penicillium* spp. produced the highest amount of invertase in the presence of 5% w/v orange peel waste as the carbon source (0.63 U/ml). The optimum incubation period for invertase production was observed after seventh day of incubation periods, using two disks 4 mm in diameter, which produced maximum invertase activity of 1.98 U/ml. The crude enzyme from *Penicillium* spp. was partially purified by 60% acetone and produced 0.44 U/ml, and then immobilized invertase by covalent binding on chitin showed the highest immobilized yield (88.1%). Overall, 5.0 mg of sucrose as substrate gave the highest activity yield. The optimum conditions for immobilized invertase were at pH 6.0, 30°C, after 30 min of the reaction time; the highest immobilization yields of invertase were 93.2, 98.1, and 100.2%, respectively. The immobilized invertase was completely stable at 50°C, for 30 min Moreover, tryptone, alumina, l-serine and hydroxyproline, zinc sulfate, and EDTA enhanced the yield of invertase immobilized with 182.7, 165.1, 137.7, 134.8, 117.6, and 108.2%, respectively.

Keywords:
agriculture wastes, immobilization, invertase, *Penicillium* spp, production

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Introduction

Invertase (β-fructofuranoside-fructohydrolase; fructofuranosidase; E.C.3.2.1.26) is an enzyme that hydrolyzes sucrose by cleavage the glycoside bond (β 1-2 linkage) from disaccharide sucrose forming mixture of monosaccharide glucose and fructose. Invertase is noncrystallizable and is ~1.5 times sweeter than sucrose. Fungi, bacteria, and yeast are capable for production invertase enzyme, which has a wide range of commercial applications in food industries and in pharmaceutical sectors [1].

Invertase is widely distributed in various microorganisms such as bacteria [2,3], fungi [4–6], and yeasts [7,8]. Alves *et al.* [9] reported that there are few results on production of invertase from molds. Most fungi that produce invertase are filamentous fungi especially from *Aspergillus* spp. [10,11], *Penicillium* spp. [12], *Rhizopus* spp. [13], and *Fusarium* spp. [14]. There is a great demand for production of invertase from filamentous fungi, which is probably owing to their biotechnological applications for the production of invert sugar, food, and beverages. Utilization of submerged fermentation for production of invertase enzyme from many cultures have been reported by many studies [15–17].

Agricultural waste as carbon source utilization in submerged fermentation is an economical process for production of industrial invertase enzyme [18]. High yield of invertase depends on the use of different varieties of agricultural wastes as substrate for invertase production [19–23].

Many years ago, immobilization process became a key parameter in improving the enzyme stability and reusability several times without enzyme loss with their resistance to different environmental factors [24,25]. The immobilization method and the carrier capacity plays a major role in the success of immobilization process [26].

Different carriers for enzyme immobilization of enzymes are an important challenge in biotechnology. The use of low-cost carriers and safety carriers allows the immobilization method to be attained successfully in industrial food field without additional production cost. Many studies were interested in the support for immobilization of invertase in different aspects such as polyacrylamide [27,28], chitosan [29], diethylaminoethyl cellulose [27], and synthetic sponge [30]. Microbial invertase are usually produced either by free or immobilized cells. Immobilized cells have been used in a variety of applications such as biosensors, biotransformation, degradation of phenol, and production of ethanol.

Materials and methods

Microorganisms

The local fungal strains *Penicillium* spp. and *Trichoderma viride* were isolated from the Egyptian soil and identified at Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Giza, Egypt. All isolates were stored on potato dextrose agar medium slants at 4°C. Isolation and purification of fungi were carried out using single spore and hyphal tips technique according to Atalla and Nour El-Din [31].

Processing of the substrates

Different agricultural wastes (potato peel, wheat bran, apple peel, orange peel, carrot peel, beet peel, and sugar cane bagasse) were used as sole carbon source and tested for invertase production. All wastes were washed, dried at 70°C in oven, and cut into small pieces before use. Chitin was obtained from products of Fluka Company, Buchs, Switzerland.

Fermentation conditions

The media used for invertase production, according to Uma *et al*. [21], consist of (g/l) different agricultural wastes 20.0, yeast 10.0, ammonium sulfate 1.0, magnesium sulfate 0.75, and potassium dihydrogen phosphate 3.5, at pH 5.5. An Erlenmeyer flask of 250 ml containing 50-ml fermentation media was inoculated with 2 disks 6 mm in diameter and incubated for 7 days at 28±1°C at 180 rpm. At the end of the incubation period, cultures were centrifuged at 8000 rpm. The cell-free supernatant was used as a crude enzyme for further determinations.

Enzyme assay

Invertase activity was determined using the method of Aranda *et al*. [32]. It was modified by incubating 0.1 ml buffer 0.9 ml of 1% sucrose in 0.03 M acetate acid pH 5.0, incubated at 50°C for 30 min. To stop the reaction, 1 ml of dinitrosalicylic acid was added in boiling water bath for 5 min. One unit of enzyme activity was defined as the amount of enzyme required for release of 1 μmol of glucose/ml/min under assay condition. The absorbance was read at 540 nm.

Production of invertase

Effect of different agricultural wastes

Different agricultural wastes (potato peel, wheat bran, apple peel, orange peel, carrot peel, beet peel, and sugar cane bagasse) were added as carbon source in fermentation media inoculated with *Penicillium* spp. And *T. viride,* each of them alone. Measurement of invertase activity was determined.

Optimization of fermentation media

Improvement of culture conditions from *Penicillium* spp. Various process parameters influencing enzyme production were studied including different concentrations of orange peel (1–10% w/v), different incubation periods (5, 7, and 10 days) and different inoculums size (mm in diameter), and determination of enzyme activity was required.

Partial purification of crude invertase

A known volume of cold acetone was added slowly to the cold enzyme solution until the required concentration of acetone was reached. After removing the precipitate fraction in refrigerator centrifuge, acetone was added to the supernatant and the process was repeated until the final concentration of acetone was reached. Determination of invertase activity was carried out.

Immobilization of partially purified invertase

Chitin, foam, and saw dust were used as carriers for invertase immobilization by covalent binding. Certain weight (0.5 g) of all carriers was mixed with 1 ml of

partially purified invertase and incubated for 1 h and then the unbounded enzyme was washed with distilled water. Determination of enzyme activity was measured [33].

The immobilization yield was calculated by the following equation:

$$\text{Immobilization yield}(\%) = (I/A - B) \times 100$$

where A=activity of added enzyme; B=activity of unbound enzyme, and I=activity of immobilized enzyme

Characterizations of immobilized enzymes

Different substrate concentrations on the activity of immobilized enzymes

Data concerning the effect of different substrate concentration (2.5, 5.0, 10.0, 15.0, 20.0, 25.0 mg/ml) on immobilized invertase activity were obtained.

Different pH values of the reaction

The effect of different pH values on the activity of immobilized enzyme was investigated using acetate buffer (0.2 M), with pH 3.5–5.5, and phosphate buffer (0.2 M), with pH 6.0–7.5.

Different temperature of the reaction

In this experiment, the effect of temperature of the reaction on invertase activity was studied using different temperatures ranging from 30 to 60°C.

Different time of the reaction

This experiment was conducted to select the most suitable reaction time for invertase activity by *Penicillium* spp. The reaction was carried out after 10, 20, 30, 40, and 50 min.

Thermal stability

In this experiment, the immobilized enzyme was incubated, in absence of substrate, at different temperatures from 30 to 60°C, each for 10–60 min; thereafter, the residual invertase activity was determined under the standard conditions.

Addition of different activator and inhibitors

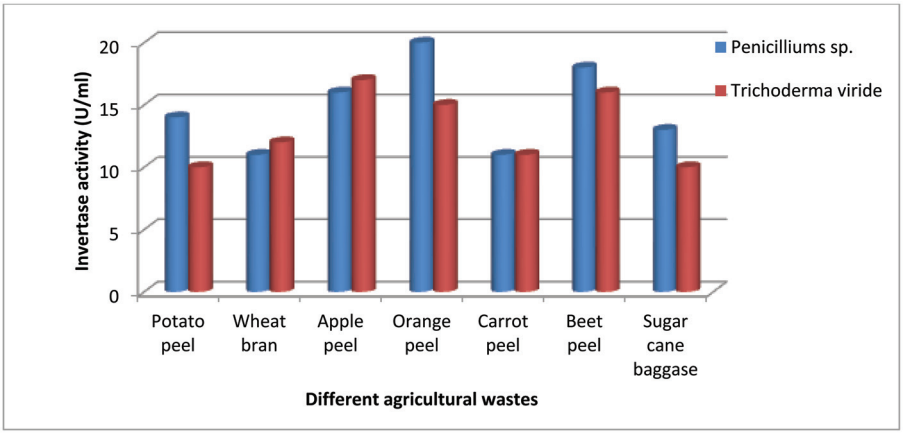
To assay the enzyme activity in the presence of some different metal ions, different activators and inhibitors such as alumina, EDTA, hydroxyproline, l-serine, tryptone, copper sulfate, magnesium chloride, zinc sulfate, ferrous sulfate, and ammonium sulfate were added at constant weight to immobilized enzyme to determine their effect on immobilization yield.

Results and discussion

Effect of different agricultural wastes on invertase production using *Trichoderma viride* and *Penicillium* spp.

Comparison between invertase production in fermentation media inoculated with *T. viride* and *Penicillium* spp., each of them alone, was done. Data recorded in Fig. 1 revealed that maximum invertase activity was obtained in the fermentation media inoculated with *Penicillium* spp. containing orange peel waste as substrate, which produced 0.22 U/ml, followed by beet peel as substrate, which produced 0.18 U/ml, and then by apple peel, which produced 0.16 U/ml, and other wastes produced moderate to low activity. In contrast, the other media inoculated with *T. viride produced* maximum activity using apple peel waste as carbon source, which produced 0.17 U/ml, followed by beet peel waste, which produced 0.16 U/ml. From the aforementioned results, it is most suitable to inoculate the fermentation media containing orange

Figure 1



Effect of different agricultural wastes on invertase production using *Trichoderma viride* and *Penicillium* spp.

peel waste as substrate with *Penicillium* spp. This result coincided with Uma et al [21] who found that pomegranate peel was a good substrate for invertase production by *Cladosporium cladosporioides*, and also Kashif *et al*. [34] found that sunflower waste was suitable for invertase production by *Aspergillus niger*. These results were not coincident with Mehta *et al*. [35] who found that fructose as carbon source was most suitable for invertase production by *A. niger*.

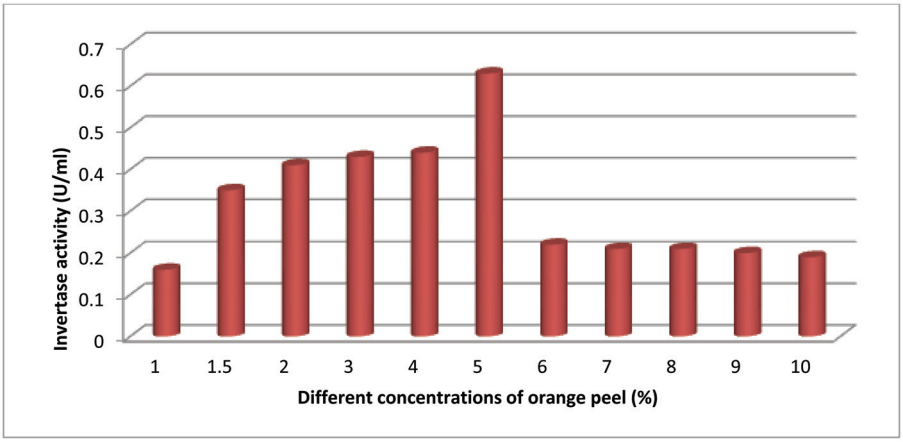
Effect of different concentrations of orange peel on invertase production by *Penicillium* spp.

Orange peel waste was known to enhance enzyme production; the effect of different concentrations of orange peel waste on invertase production using *Penicillium* spp. was studied. Results in Fig. 2 showed that the maximum invertase production (0.63 U/ml) was found with orange peel waste at concentration of 5%w/v. Further decrease or increase in the orange peels concentration decreased extracellular invertase production. Similar results were recorded by Shankar *et al*. [36], who studied the effect of different concentration of orange peel on invertase production using *Saccharomyces cerevisiae* MK and showed maximum production of invertase at ~4% supplemented medium, whereas minimum amount of invertase production in 10% supplemented medium.

Effect of different incubation periods on invertase production

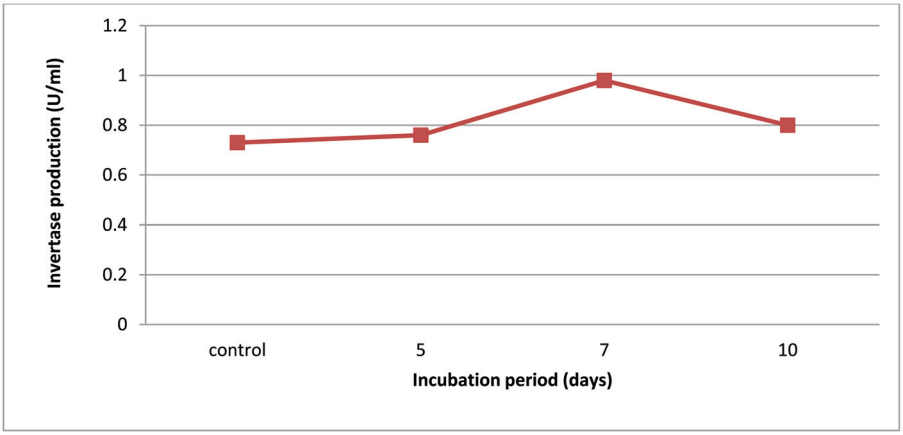
To determine the effect of incubation period on invertase production by *Penicillium* spp., the results presented in Fig. 3 reported that the maximum amount of the invertase production (0.98 U/ml) was observed on the seventh day of incubation. Further increase in incubation period gave less invertase production. This result is in contrast with Lincoln *et al*. [37] where the highest levels of invertase activity produced from *Aspergillus* sp. were observed at 72–96 h of incubation. Moreover, *Aspergillus nidulans* and

Figure 2



Effect of different concentrations of orange peel on invertase production by *Penicillium* spp.

Figure 3



Effect of different incubation periods on invertase production from *Penicillium* spp.

Emericella nidulans cultures were reported with maximum activity at 72 h of incubation [9]. In another instance, Chen *et al.* [38] found that *A. nidulans* and *Aspergillus caespitosus* reported highest activity on the third day of incubation. Invertase production by *Paecilomices variotii*, a filamentous fungus, showed highest activity after 72 h of incubation [39]. However, *Mucorgeophillus* EFRL 03 produced optimum enzyme activity at 48 h [40].

Effect of different inoculums size on invertase production

The results recorded in Fig. 4 showed that invertase activity increased gradually till reached 1.98 U/ml in fermentation media inoculation with two disks 4 mm in diameter produce followed by two disks 6 mm in diameter, which produced 0.98 U/ml. Other disks used for inoculation produced minimum to low activity. These results were the same with Ul-Haq and Ali [41] who discussed that increase in the inoculum size led to increase in production of the enzyme regularly and then decreased. Productions of invertase from *C. cladosporioides* reached its maximum activity at 3% inoculum size using pomegranate peel as substrate [21]. The same finding was observed for invertase production from *Aspergillus flavus* using orange peel was at 3% of inoculum size produced 25.8 U/ml. Inoculum more than the optimum of *A. flavus* caused overgrowth and nutrient imbalance, resulting in less enzyme production [20]. Amount of inoculum had a certain effect on invertase production [42]. At low inoculum size, the cells extant in the culture might not be enough to employ enough amount of substrate to produce enzyme. However, at high inoculum size, viscosity of fermentation medium might increase owing to high growth of fungi resulting in excessive uptake of nutrients, nutritional imbalance in the medium or before the cells in the culture were physiologically ready to start enzyme production [43].

The effect of different kinds of incubation time was tested on invertase production. The maximum amount of invertase production was observed in 48-h incubation time (0.36±0.015 IU/ml). The minimum amount of invertase production was obtained in 96-h of incubation time (0.08±0.004 IU). The effect of different kinds of incubation time was tested on invertase production. The maximum amount of invertase production was observed in 48-h incubation time (0.36±0.015 IU/ml). The minimum amount of invertase production was obtained in 96 h of incubation time (0.08±0.004 IU)

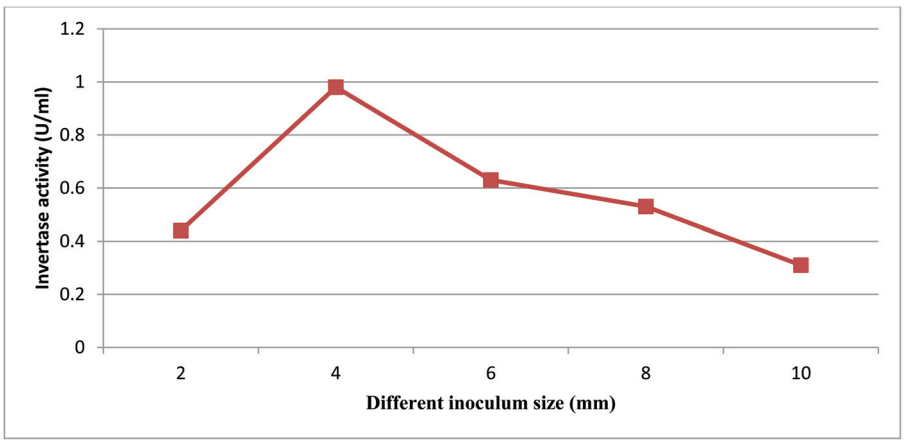
Partial purification

The crude enzyme was partially purified using acetone fractionation (60%) v/v. The partially purified enzyme was assayed for invertase activity, which was found to be 0.44 U/ml.

Invertase immobilization

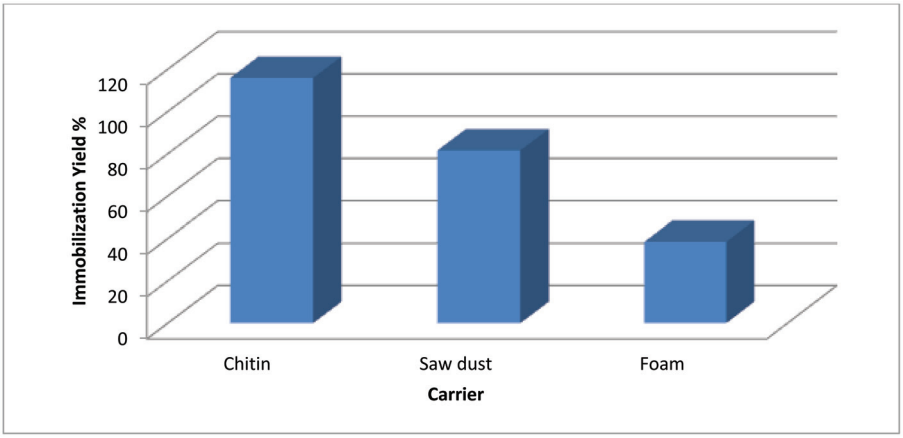
The partially purified invertase was immobilized by covalent binding on different carriers. Data presented in Fig. 5 indicated that invertase immobilized by covalent binding on chitin showed the highest immobilized yield (88.1%) followed by saw dust, which gave the immobilization yield of 81.5%. A lower immobilization yield was obtained by covalent binding on foam (38.3%). In immobilization studies, the mode of attachment of the enzyme to the carrier and the carrier characteristics play an important role in defining the properties of the bound enzyme [44]. Doaa *et al.* [45] found that immobilized invertase from *S. cerevisiae* on foam, active carbon, and

Figure 4



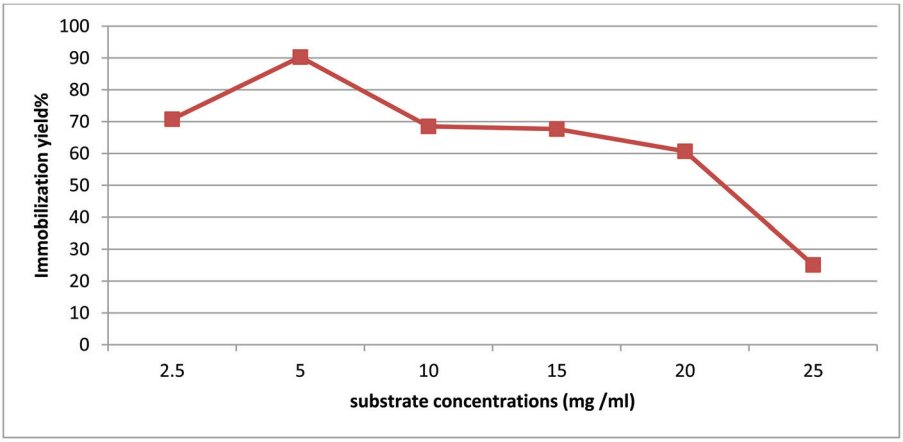
Effect of different inoculums size on invertase production by *Penicillium* spp.

Figure 5



Immobilization of invertase on different carriers.

Figure 6



Effect of different substrate concentrations on the activity of immobilized invertase.

Dowex not retain any invertase activity, whereas matrix like lufa, sponge, and wool gave low immobilization yield, although they are characterized by high porosity but immobilization yield were 10, 25, and 30%, respectively. Moreover, cellulose and wood sawdust gave the highest immobilization yields of 80 and 84% respectively. Esawy *et al.* [46] reported the immobilization yield from 58.9% in PVA sponge alone to 71% in PVAsp Gs. Silica gel gave considerably high immobilization yield of 80% [47].

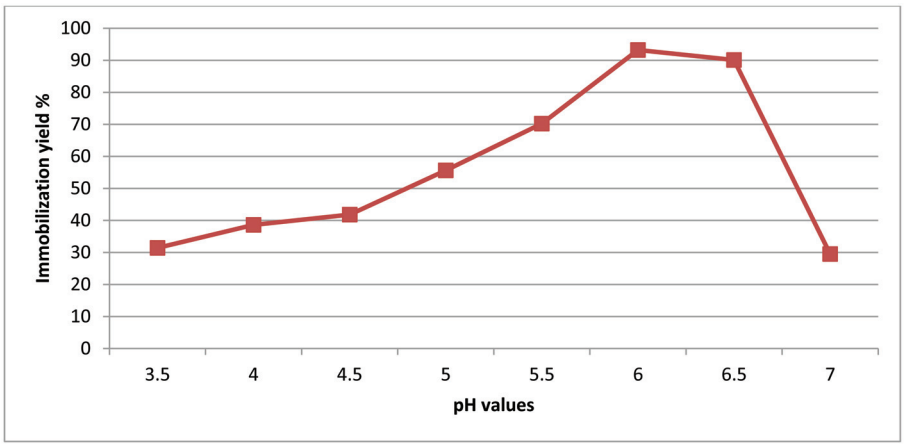
Characterizations of immobilized enzymes

Effect of substrate concentrations

The effect of different concentrations of sucrose on activity of immobilized invertase produced from *Penicillium* spp. was studied. Results in Fig. 6 showed that immobilization yield increase gradually till reached 90.3% immobilization yield at 5.0 mg of sucrose, and then activity decreases. Mouelhi *et al.* [48] found that 2% molasses allowed the highest activity yield of 64%. Moreover, 1% of molasses also permitted synthesis with 59% of activity yield. A high concentration of molasses with high viscosity caused less decrease in the activity of immobilized invertase from *Sclerotinia sclerotiorum*. For the native enzyme, the saturation was observed at 0.8 mg/ml, whereas for immobilized enzyme, it was observed at 0.9 mg/ml. There is no significant difference in the saturation concentration of both the immobilized and native enzyme. The slight difference in the saturation concentration may be owing to the availability of active sites for the binding of the substrates [49].

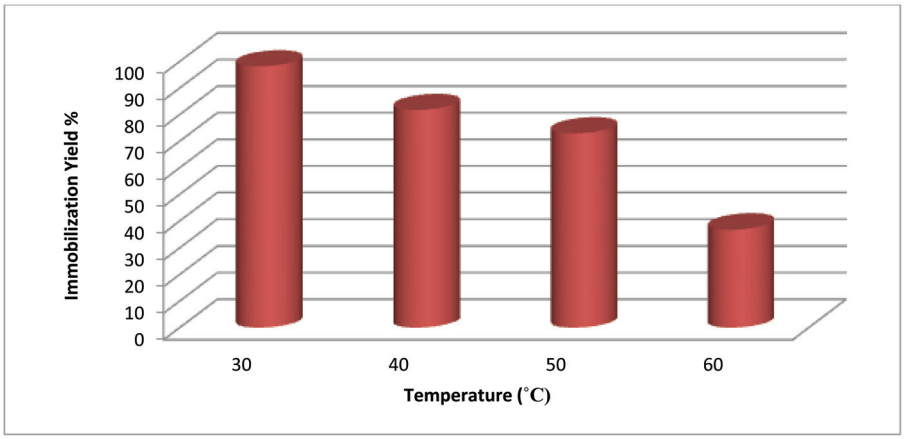
It was found that 2% molasses allowed the highest activity yield (64%). Moreover, 1% of molasses also permitted synthesis with 59% of activity yield. A high concentration of molasses repressed activity of the immobilized invertase, as well as the use of 3, 4, and 5% allowed depression of activity (38.15, 30 and 14.89%, respectively).

Figure 7



Effect of different pH values.

Figure 8



Effect of different temperature on immobilized invertase.

Effect of different pH values

The data recorded in Fig. 7 showed that immobilization yield increase till reached pH 6.0, which produced maximum activity of 93.2% immobilization yield, followed by pH 6.5, and then activity decreased. This result coincided with Basha *et al.* [50] who found that immobilized of invertase from *Thermomyces lanuginosus* was optimum at pH 6.0. Immobilization of invertase from yeast was at pH 4.0 [51]. The maximum activity of immobilized invertase from *C. cladosporioides* occurs when at pH 6.0 [52].

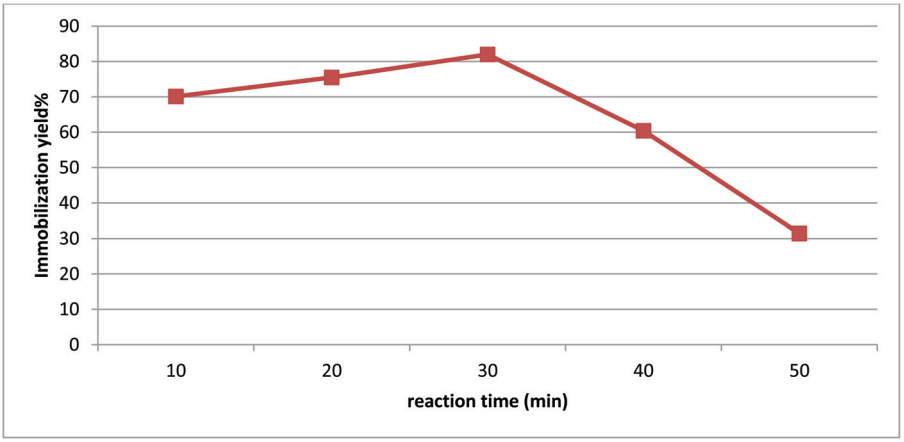
Effect of different temperature

Results in Fig. 8 showed that the 30°C was recorded as the optimal temperature for immobilized invertase enzyme with 98.1% immobilization yield; with more than 30°C, the activity decreased. These results were in contrast with Khobragade et al [49] who found the optimum temperature for immobilized invertase was observed at 50°C. The maximum activity for immobilized invertase from *S. cerevisiae* was 70°C [53]. The immobilized invertase obtained by *T. lanuginosus* presented its maximal activity at 50°C. This result determined that in the presence of substrate, the enzyme appears to be inactivated faster at higher temperatures [50].

Effect of different time of the reaction

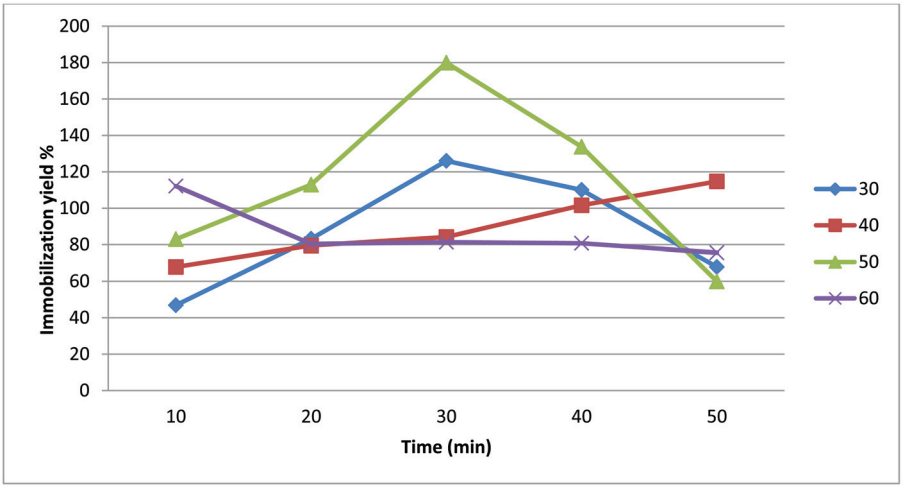
The data recorded in Fig. 9 showed that immobilization yields of invertase increased gradually till it reached 100.2% after 30 min of the reaction, and then the activity decreased. Lessiy *et al.* [54] found that maximum invertase from baker yeast increased from 5 to 30 min after immobilization. The optimum reaction time of immobilized invertase produced from *A. niger* EM77 was after 15 min of the reaction [46]. The immobilized invertase from *T. lanuginosus* reaction time was linear up to 40 min [50].

Figure 9



Effect of the time of the reaction on the activity of immobilized invertase.

Figure 10



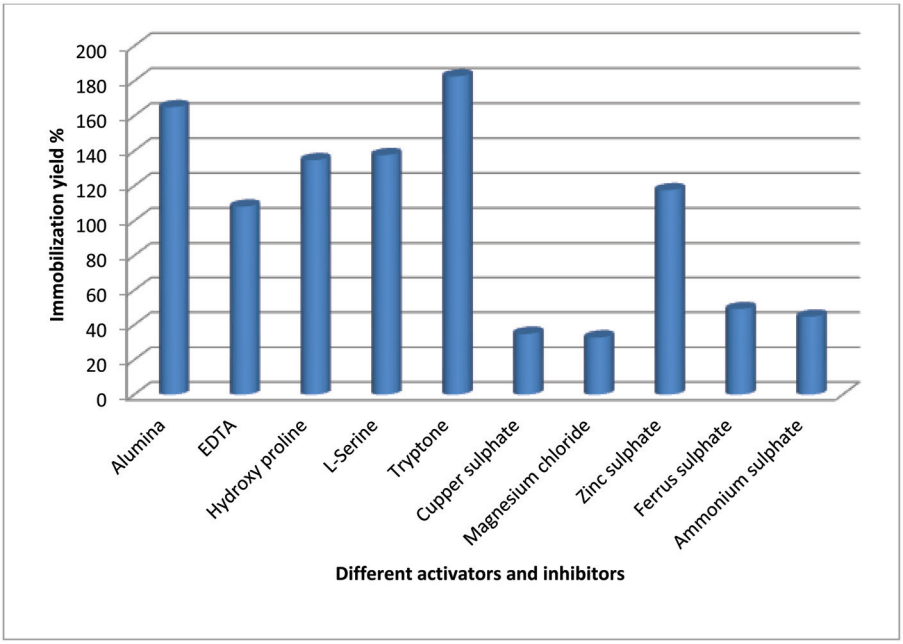
Thermal stability of immobilized *Penicillium* spp. invertase.

Thermal stability

Thermal stability is one of the most important parameters of the improved biocatalysts. The results in Fig. 10 reported that the immobilized invertase was completely stable at 50°C; immobilization yields increase till reached 179.9% after 30 min, and then the activity decreased. Moreover, a decrease in the stability of the immobilized invertase was observed. The thermal stability of immobilized invertase was 126% immobilization yield after 30 min at 30°C, 114.7% immobilization yield after 50 min at 40°C, and 112.1% immobilization yield after 10 min at 60°C. Similar findings were reported for immobilized invertase by Basha *et al.* [50] which found that immobilized invertase from *T. lanuginosus* retained approximately 83% of the activity after 3 h at 50°C. Aleksandra and Zoran [53] found that the thermal stability was 65% higher at 50°C, 47% at 60°C, and 42% at 70°C. Moreover, the immobilized invertase produced from *A. niger* EM77 kept 80% of its original activity at 70°C after 45 min [46].Effect of different activators and inhibitors on activity of immobilized invertase enzyme

The results recorded in Fig. 11 showed that tryptone, alumina, L-serine, hydroxyproline, zinc sulfate, and EDTA enhanced the yield of invertase immobilized with 182.7, 165.1, 137.7, 134.8, 117.6, and 108.2%, respectively. Considerable decrease of activity was observed with magnesium chloride and copper sulfate. The immobilized invertase was more resistant to inhibition by metal ions: Mn^{+2} inhibited completely, and Zn^{+2}, Fe^{+3} and Cu^{+2} inhibited the immobilized invertase to 82, 63, and 67%, respectively. Ca^{+2} inhibitions was 30% for the immobilized invertase [50]. Uma *et al.* [21] found that Zn^{+2} and Cu^{+2}

Figure 11



Effect of activators and inhibitors on activity of immobilized invertase.

inhibited enzyme activity of the fungal strain *A. flavus*. This means that invertase activity inhibited while incubating with highly heavy metal ions. The glycosylation of enzyme formed stable covalent bond which led to success of resistance against chemicals [33]. The major low activity of free enzyme may be attributed to direct contact between metal ions and active site of enzyme. However, in immobilized enzyme, the fibrous porous structure plays a role of protection owing to the time required for these metal ions (Cu^{+2}, Hg^{+2}, and Fe^{+3}) to diffuse the carrier surface till reach the active site of enzymes. These results indicate that partial protection of the enzyme by immobilization, which are in agreement with those reported for other immobilized enzymes [55].

Conclusion

Production of invertase from *Penicillium* spp. using 5 mg of orange peel as carbon source after 7 days of incubation period using two disks 4 mm diameter produced optimum enzyme activity. Invertase produced from *Penicillium* spp. was partially purified by 60% acetone and successfully immobilized by covalent binding on chitin. The highest immobilization yield of invertase obtained using 5 mg sucrose at pH 6.0, 30°C, after 30 min of the reaction. The immobilized invertase was completely stable at 50°C for 30 min. Different metal ions were tested to activate or inhibit immobilized invertase.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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