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Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes

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ABSTRACT

The *Aspergillus niger* NRC1ami pectinase was evaluated according to its hydrolysis efficiency of dry untreated orange peels (UOP), HCl-treated orange peels and NaOH-treated orange peels (HOP and NOP). Pectinase was entrapped in polyvinyl alcohol (PVA) sponge and the optimum pH and temperature of the free and immobilized enzymes were shifted from 4, 40 °C to 6, 50 °C respectively. The study of pH stability of free and immobilized pectinase showed that the immobilization process protected the enzyme strongly from severe alkaline pHs. The immobilization process improved the enzyme thermal stability to great instant. The unique feature of the immobilization process is its ability to solve the orange juice haze problem completely. Immobilized enzyme was reused 12 times in orange juice clarification with 9% activity loss from the original activity. Maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) of the partially purified form were significantly changed after immobilization.

1. Introduction

Pectinases are heterogeneous groups of enzymes catalyzing the degradation of the cell wall pectin component in plant and are produced by various fungi including *Aspergillus* sp. (Martinez-Trujillo, Arreguin-Rangel, Garcia-Rivero, & Aguilar-Osorio, 2011), *Penicillium* sp. (Teixeira, Gonçalves, de Queiroz, & de Araújo, 2011), and *Thermoascuss aurantiacus* (Martins, Silva, da Silva, & Gomes, 2002). Several studies have been achieved to produce pectinase by fungi using citrus peels, sugar cane molasses and other agricultural wastes as substrates (Kareem & Akpan, 2004). Pectinases have a wide range of technical applications including simultaneous saccharification of citrus peel wastes into simple sugars, oligosaccharides and bioethanol (Wilkins, Widmer, & Grohmann, 2007). Pectinases have also been used commercially to aid in extracting juice from fruit. The role of pectinases is well known in reducing the cloudiness of the juice caused by suspended pieces of cell wall (Ismail, 1996). The use of pectin lyase alone, instead of pectin methyl esterase for fruit juice clarification, prevents the release of methanol in the juice, which constituting a potential health hazard in non-concentrated juices (Szajer & Szajer, 1982). Moreover, the volatile ester content, responsible for the specific aroma of various fruits, is not damaged (Alana, Alkorta, Dorminguez, Liama, & Serra, 1990).

An increased interest was focused for the preparation of immobilized pectinase for clarification and depectinization of fruit juice applying a wide variety of carriers and methods (Alkorta, Suarez, Herranz, Gonalez-muniz, & Garcia-Lopez, 1996). Supports suitable for technical applications should maintain a high level of enzyme activity, while preventing a possible leaching out during the reaction (Lei & Bi, 2007).

Several methods have been developed for the preparation of immobilized pectinase. Each has its own advantages and disadvantages (Li, Li, Wang, & Lirui, 2008). The synthetic polymers such as, polyacrylonitrile (Godjevargova & Gabrovska, 2003) and nylon (MohyEldin, Schroen, Janssen, Mita, & Tramper, 2000), could be used for enzyme immobilization, but on contrary to the natural macromolecules (Krajewska, 2004) they have some disadvantages, as the imperfect biocompatibility and hydrophobicity.

Entrapment immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. The feasibility of using synthetic sponge as a carrier for immobilization was reported by many authors (Hanaki, Hirunmasuwan, & Matsuo, 1994). The lattice structure of polyvinyl alcohol (PVA) sponge characterized by very dense porosity and specific pore volume are very

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high. These properties recommended PVA sponge strongly to be used for the enzyme entrapment.

The present article studied the *Aspergillus niger* NRC1ami pectinase production in the presence of the Egyptian citrus peels as the sole carbon source. The saccharification efficiency of the partially purified pectinase was tested through the untreated orange peels (UOP) and the treated orange peels (HOP and NOP). The partially purified pectinase was immobilized by entrapment within polyvinyl alcohol sponge. The influence of sponge size and enzyme loading was studied. Immobilized enzyme properties such as: temperature, pH, thermal stability, pH stability and its operational stability were studied. The role of the free and the immobilized enzyme forms in orange juice clarification was evaluated.

2. Experimental

2.1. Fungus isolation

The fungus was isolated from Egyptian orange fruit (Bousorra), purchased from the local market in Cairo city. Small parts of orange rut were gently shaken in 250 ml flask containing 100 ml sterilized distilled water for 15 min on a mechanical wrist action, then serial dilutions were usage. One ml of the desired dilution was transferred aseptically over the surface of Petri-dish containing appropriate amount of potato-dextrose medium and was streaked above agar surface. After incubation within 4–7 days at 30 °C, one colony was picked up by sterilized needle and re-cultivated on potato-dextrose medium. The cultivation was repeated 3 times and the purity of the colony was examined under visible microscope.

2.2. Fungus identification

The fungus was identified as *A. niger* NRC1ami in Cairo University, Micro-analytical center, Giza, Egypt.

2.3. Production medium

The following medium was reported by (Ismail, 1996) with some modification and had the following composition (g/l): Egyptian citrus peel 15, K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; yeast extract, 20, the pH of the media was 5.0.

2.4. Inoculums preparation and culture conditions

This was done by addition of 5 ml sterile production medium to each of two 4-days-old slants scratched with sterile needle. One ml of spore suspension was spread on Petri-dish containing solidified potato-dextrose medium. After 4 days incubation at 30 °C, the cultivated medium was cut into equal discs with sterilized cork borer and two disks was transferred to each 250 ml Erlenmeyer flasks containing 50 ml of the sterilized production medium. The flasks were then incubated for 4 days in rotator shaker (at 150 rpm) at 30 °C. The culture broth from each flask was filtered off and the culture filtrate was then centrifuged at 4000 rpm for 10 min in a cooling centrifuge (JANEKTZKI, K 70, Germany). The filtrate was used for protein content and enzyme activity determination.

2.5. Pectinase assay

The pectinase activity was determined using 1% (w/v) citrus pectin as substrate product of Fluka company Switzerland (0.3 ml of enzyme + 0.7 ml substrate for free enzyme for 15 min at 40 °C or one piece of sponge +1 ml substrate for 15 min at 40 °C in case of immobilized form), The liberated galacturonic acid content was determined by the Somogyi-Nelson method (1952).

One unit (U) of pectinase activity was defined as the amount of enzyme producing 1 μmol galacturonic acid per min.

2.6. Viscometric method

Both free and immobilized pectinase activities were determined by using viscometric method (the percentage decrease in relative viscosity of 0.5% pectin solution (w/v) calculated according to the following formula (White and Fabian, 1953)):

$$\frac{A-B}{A} \times 100$$

where A = time of flow in seconds of inactivated reaction mixture (blank). B = time of flow in seconds of the active reaction mixture.

2.7. Protein determination

This was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.8. Orange peel dryness

The untreated orange peels (UOP) were washed with large excess of hot water (60–70 °C) and the process was repeated until no sucrose or fructose remained in the residual solids. The extracted solids were filtered and dried at 50 °C. The pretreated peels were prepared by adding 0.1 N NaOH or 0.1 N HCl separately to the dried orange peels, after 1 h the treated peels was washed with excess of distilled water, filtered and dried again.

2.9. Enzymatic saccharification

This was conducted by incubation of the enzyme and the dry crushed untreated orange peels (UOP), NaOH-treated orange peels (NOP) and HCl-treated orange peels (HOP) in 0.2 M acetate buffer, pH 4.0 in a water bath at 40 °C for 24 h. The mixture was filtered and the residual of orange peels was washed, dried at 50 °C and weighed. The rate of hydrolysis of 1 g of dry orange peels was calculated.

2.10. Quantitative determination of the hydrolysis products

Chromatographic separation of the hydrolysis products was carried out on Whatman No. 1 filter paper, applying the solvent system n-butanol–acetone–water (4:5:1, v/v/v) according to (Jayme & Knolle, 1956) method as follows:

After chromatographic separation, the chromatogram was air dried and dipped in 40–50 ml of the color reagent, air dried and then heated at 105 °C for 10 min in an oven for developing the spots. The individual spots were cut off, divided into small strips and dropped into test tubes containing 4 ml of an eluting agent and shaken for complete elution. The absorbance at 390 nm of the color solution was determined in a Bausch & Lomb Spectronic 2000 spectrophotometer, for hexoses, and at 360 nm for pentoses. The quantities of sugars were determined by applying the standard curves constructed under the same conditions according to Wilson, Nessler, and Paul (1959).

2.11. Determination of total carbohydrates

Total carbohydrate was measured by the phenol–sulfuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) with galacturonic acid (GalA) as standard. In hot acidic medium glucose is dehydrated to hydroxyl methyl furfural forming a yellowish orange color of maximum absorption 490 nm with phenol.

2.12. Fractional precipitation with ethanol

Ethanol (30–50–70–90%) was added slowly to the ice-cold enzyme solution until the required concentration of the ethanol was reached. After removing the precipitated fraction by centrifugation in a refrigerated centrifuge at 4000 rpm for 10 min, further ethanol was added to the supernatant fluid and the process was repeated until the final concentration of ethanol was reached (90%). Many enzyme fractions obtained at 30, 50, 70 and 90% ethanol concentrations were dried over anhydrous calcium chloride, under reduced pressure at room temperature, weighed and used for pectinase activity and protein estimation.

2.13. Enzyme immobilization by entrapment

The method was modified from Esawy, Abdel-Fattah, and Mahmoud (2008). Polyvinyl alcohol sponge (PVA) was washed several times by distilled water, squeezed to eject water and was cut into small pieces (5 mm × 5 mm × 2 mm) using scissors. The pieces of synesthetic sponge were inserted into the 50% ethanol enzyme fraction dissolved in 2.0 ml of 0.2 M acetate buffer, pH 4.0 at 4 °C for 24 h (1037 U/g carrier).

2.14. Optimization parameters for immobilized pectinase

The parameters studied included sponge size from 5 mm × 5 mm × 2 mm to 30 mm × 30 mm × 2 mm and enzyme concentration from 0.05 to 0.25 g/5 ml.

2.15. Optimum pH and temperature

In the present experiment, identical reaction mixtures were incubated at different temperatures (30–70 °C) for 15 min at pH 5. The enzyme assay was carried out as discussed previously. The optimum reaction pH was determined at the range of 4–10 at 50 °C for 15 min also and the enzyme assay was done as shown previously.

2.16. pH and thermal stability

The pH stability of the free and immobilized enzyme forms was examined after pre-incubating the enzyme samples at the optimum temperature (50 °C) for 15–120 min at different pHs (4–9) applying the appropriate buffer solutions.

At the end of heating period, the pH was readjusted to the value of the standard assay. Then the residual activity was determined, applying the standard conditions. The thermal stability of the enzyme samples was examined by dissolving in acetate buffer (0.2 M, pH 4.0) and incubating in water path at different temperatures (40–70 °C) for 30–120 min and the residual activity was determined applying the standard conditions.

2.17. Immobilization yield (U/g carrier)

It was calculated according to the following equation:

$$\text{Immobilization yield}\,(\%) = \frac{\text{Immobilized enzyme activity}(I)}{\text{enzyme added activity}\,(A) - \text{Unbound enzyme activity}\,(B)} \times 100$$

2.18. Orange juice clarification

Egyptian mature orange fruits (Balady) obtained from a major market in Cairo, Egypt were washed and peeled. Orange juice 20 g was pasteurized at 85 °C for 3 min to inactivate the fruit enzymes or microbes, after cooling, 1 ml of the partially purified pectinase (14.7 U) was added with stirring and the mixture was incubated in water bath. This setup (after stirring) was achieved at 50 °C for 24 h. With the aid of coffee filter paper the juicy part was filtered and the pectinases activity on the orange juice was determined. Distilled water was replaced instead the active enzyme in the control.

2.19. Detection of pectin methyl esterase

This was carried out according to the method of Smith (1958) with some modifications. The orange juice pH was determined by litmus paper and then the immobilized pectinase (0.1 g) or the free form (14.7 U/ml) was added. Hydrolysis of ester linkages by pectin methyl-esterase results in a gradual acidification, which in turn results in development of yellow color.

2.20. Operational stability

It was performed with 0.1 g of wet immobilized pectinase (containing about 103 U of the enzyme) incubated with 5 ml orange juice at 50 °C for 15 min. At the end of the reaction period, it was removed by filtration, washed with distilled water and resuspended in 5 ml of freshly prepared juice to start a new run and this process repeated 12 times. In all cases the supernatant was assayed for pectinase activity.

2.21. Enzyme kinetics and activation energy

For the determination of Michaelis–Menten kinetic parameters of the enzymes forms, different initial pectin concentrations were used (0.25, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5%). The activation energy of the free and immobilized pectinase was calculated from the Arrhenius plot. It was determined from the slope of linear representation of $\log(V_M)$ according to $1/T$, resulting from the Arrhenius law:

$$\log(V_M) = \frac{Cte - E_A}{RT}.$$

The enzyme activity (V_M) was expressed in mol min^{-1}, the temperature (T) in degree Kelvin (K), the gas constant ($R = 1.987$) in cal K^{-1} mol^{-1}, and the activation energy (E_A) in cal mol^{-1}.

2.22. Statistical analysis

Data analysis was carried out with Microsoft Excel (2007). All data are presented as mean ± standard error of means. The acid and alkali tolerance experiments, the bile and pancreatic enzyme tolerance were independently replicated 2 times ($n = 2$), with 2 measurements per replicate. The mean of the repeated measurements yielded the value for each replicate.

3. Result and discussion

3.1. Pectinase production from the isolate A. niger NRC1ami

In the present study orange peel isolate *A. niger* NRC1ami was cultivated on the Egyptian Bousorra orange peel waste as the sole carbon source. Looking for inexpensive media for active pectinases production aiming to their use in juice and wines industries was reported by many authors (Teixeira et al., 2011). On the other hand, Kareem and Adebowale (2007) deduced that the use of citrus peels as substrate for pectinase production would promote local production of food enzymes.

3.2. Partial purification of A. niger NRC1ami

The enzyme was partially purified using different concentrations of ethanol. The enzyme fraction precipitated at 50% ethanol

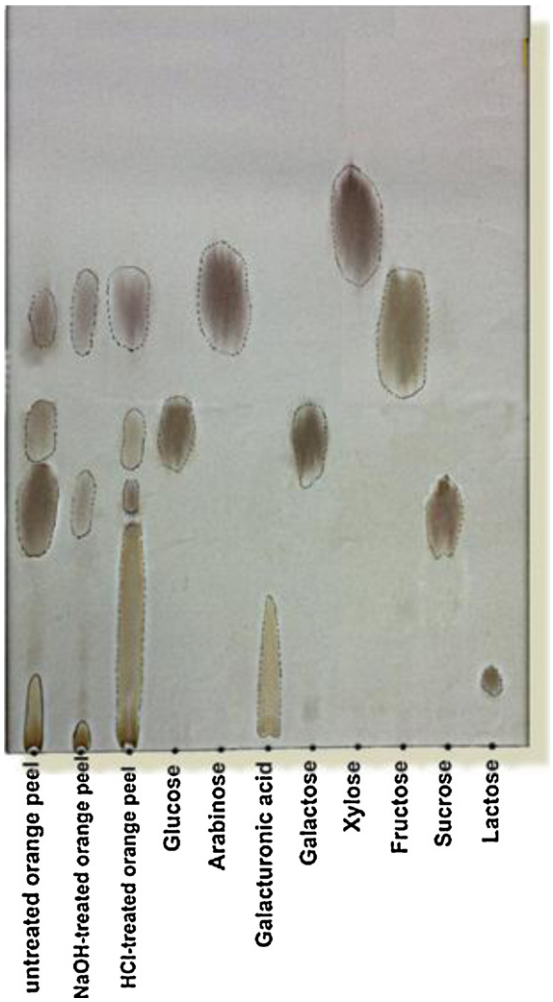

Fig. 1. Hydrolysis products of untreated (UOP) and treated orange peels (NOP and HOP) by *Aspergillus niger NRC1ami24.*

recorded that the specific activity of the crud pectinase shifted from 10.94 U/mg to 58 U/mg. This fraction affording the highest purification folds (5.3) and recovered activity (31.36%). It was used in the following experiments.

3.3. Pectinase activity measure

Saccharification process was a good criterion to pectinase activity and for determination the enzyme efficiency in many industrial applications. In this study, saccharification test was conducted to hydrolyze 1 g of the untreated dry orange peel (UOP) and treated orange peels (NOP and HOP) by the partially purified pectinase. The highest enzyme activity and carbohydrate content (Table 1) was obtained by the peels treated with 0.1 N NaOH (NOP) (583.7 U/ml and 18.5%) respectively. Also about 77% of the solid orange peel was solubilized. On the other hand, the treated with 0.1 N HCl (HOP) led to 459.5 U/ml enzyme activities and 10.2% carbohydrate content. The untreated peels afforded the lowest pectinase activity (140 U/ml) and carbohydrate content (7.4%). In this respect, Okeke (1995) concluded that the pretreatment of substrates resulted in increase of the accessible substrate surface area which significantly increased its susceptibility to hydrolysis (Kumar, Barrett, Delwiche, & Stroeve, 2009). The chromatographic analysis proved that arabinose, galactose, and galacturonic acid were the main monosaccharides with all peel samples applied (Table 1 and Fig. 1). The highest galacturonic acid was obtained with HOP and NOP (16.8 and 17%, respectively) and the lowest one was registered (3.3%) with UOP (Table 1). Similar results were obtained by Grohmann and Baldwin (1992), where the main enzymatic hydrolysis products of orange peels were glucose, galacturonic acid, galactose and arabinose.

3.4. Immobilization of A. niger NRC1ami pectinase

There are many applications for the immobilized pectinase for the clarifications and depectinization of fruit juice using a wide variety of carriers and methods (Alkorta et al., 1996). Accordingly the partially purified pectinase was entrapped in polyvinyl alcohol (PVA) sponge. The results recorded 66% immobilization yield with the enzyme dose 12.2 mg/g carrier. The specific activity of the immobilized pectinase (270 U mg^{-1}) was greatly higher than the free form (85 U mg^{-1}) and accordingly the purification fold increased 3.2. This result could be considered as partial purification step and pointed to the sponge structure which helped in entrapment most of the pectinase letting the undesired protein abroad. In this concern, the specific activity of the immobilized starch phosphorylase on hen egg shell increased from 0.023 to 0.045 U mg^{-1} (Garg & Kumar, 2008). To optimize the immobilization procedure, two variables influence the interaction between the enzyme and support were investigated. The results showed that almost all the enzyme activity was retained on the support at 20 mm × 20 mm × 2 mm sponge size and 30.5 mg protein/g carrier and the activity decreased gradually below and above these values (data not shown). This result suggested that the high porosity of PVA sponge worked like corridors of very narrow pipe helping the enzyme to entrap strongly with great efficiency. This immobilization yield was higher than that reported for pectinase alginate system (66%) by Li, Wang, Li, Zhao, and Guo (2007) and pectinase immobilized on synthetic polymers (74%) by Lei and Bi (2007).

3.5. Properties of the immobilized A. niger NRC1ami pectinase

The immobilized pectinase properties were studied in Table 2 and the optimal reaction pH shifted from 4 to 6 indicating good protection of the enzyme activity in the alkaline range. This result was similar to that of the pectinase immobilized on a chitosan support (Li, Zhong, Xiao, Ge, & Guo, 2002). Zhongli and Shuxian (2007) reported that the pH shift depends on the enzyme reaction as well as on the structure and the charge of the matrix. The pH stability (Table 2) showed that the immobilization process protected the enzyme in the alkaline range to great extent. This result could be related to the immobilization effect on the enzyme structure molecule and its conformational stability. Recently, there are many reports on the application of alkaline pectinases in the textile industry for retting and degumming of fiber crops, production of good quality paper, fermentation of coffee and tea, oil extractions and treatment of pectic waste water (Kashyap, Vohra, Chopra, & Tewari, 2001). On the other hand, the immobilized pectinase revealed acceptable pH stability over a broad experimental range (Li, Li, Wang, & Lirui, 2007). The optimal reaction temperature for the immobilized enzyme changed from 40 °C to 50 °C (Table 3) and this was accorded with those reported by (Li et al., 2008) and (Lei & Bi, 2007) who found that the optimum temperatures for free and immobilized pectinase were obtained at 50 °C. The results also revealed that the immobilization process gained the enzyme rigidity against heat inactivation (Table 3). The free enzyme lost its activity completely after 2 h at 70 °C; on the other hand the immobilized enzyme retained 52.9% pectinases activity at the same conditions. This thermal stability behavior could be due to the immobilization protection on the enzyme molecule against the conformational changes and lower flexibility, other than the multi-attachments to the support (Tardioli, Zanin, & Morae, 2006).

Table 1
Hydrolysis products of dry orange peel (UOP, NOP and HOP) by *Aspergillus niger NRC1ami24* pectinase.

Samples	Sugar percentage (%)								Total. carbohydrate (%)	Solubility (%)	Enzyme activity (U/ml)
	Glucose	Arabinose	Galactose	Galacturonic acid	Sucrose	Xylose	Fructose	Lactose			
UOP	4.1	8.5	17.1	3.3	Trace	–	Trace	–	7.4	33	140
NOP	–	35.9	24.3	16.9	Trace	–	Trace	–	18.5	77	583
HOP	16.2	10.7	4.3	16.8	–	–	–	–	10.2	48	459.6

N.B. ± standard error of means located between 0.7 and 2.2%

Table 2
Effect of pH optimum and stability for free and immobilized pectinase activity by *Aspergillus niger* NRC1ami.

pH	Optimum pHs		Free enzyme				Immobilized enzyme			
	Free	Immobilized	Exposure time (min)							
	Relative activity (%)[a]		30	60	90	120	30	60	90	120
Control	100	100	100[a]	100	100	100	100	100	100	100
4	61.6	139.7	100	100	100	100	100	100	100	100
5	51.4	159.9	97.1	61.2	55.4	21.3	100	100	100	100
6	50.1	113.1	72.9	54.5	30.6	29.2	100	100	100	100
7	46.2	95.9	30.6	26.2	24.8	7.6	100	100	100	100
8	40.3	92.2	21.9	21.3	19.8	19.5	100	100	100	100
9	100	100	20.0	19.8	18.4	17.8	100	95.8	91.4	88.7

N.B. ± standard error of means located between 0.5 and 1.7.
[a] Relative values of pectinase activity expressed in %, and obtained by Berridge's method.

Table 3
Effect of temperature and thermal stability on free and immobilized pectinase activity by *Aspergillus niger* NRC1ami.

Temp (°C)	Optimum (Temp) (°C)		Free enzyme				Immobilized enzyme			
			Exposure time (min)							
			30	60	90	120	30	60	90	120
	Free	Immobilized	Relative activity (%)[a]							
Control*	Relative activity (%)[a]		100[a]	100	100	100	100	100	100	100
40	100	100	100	91.7	88.3	83.4	100	100	100	100
50	86.8	108.1	63.5	50.8	49.8	25.3	100	97.2	95.7	93.7
60	60.8	75.1	40.2	22.5	4.03	2.7	75.5	58.8	57.6	55.0
70	58.4	63.5	24.9	17.7	Zero	Zero	59.2	57.7	53.4	52.9

N.B. ± standard error of means located between 0.3 and 1.1.
* Control: with (0.5 g/l) $MgSO_4 \cdot 7H_2O$ & (0.5 g/l) KH_2PO_4 & (2 g/l) baker's yeast at 40 °C and pH 4.
[a] Relative values of pectinase activity, expressed in %, and obtained by Berridge's method.

In addition Li et al. (2008) reported on the marked improve of the immobilized pectinase thermal stability compared with the free enzyme at 65 °C.

3.5.1. Kinetic studies

The activation energy of the free and immobilized enzyme shifted from 5.1 to 2.7 kcal mol^{-1} (Fig. 2). This result could be explained that the increase in optimum pH and temperature affect the chemical bonding within the molecules, therefore changing the shape of the active site and substrate, accordingly the reaction rate increased and the activation energy shifted. Nevertheless, Sarioglu, Demir, Acar, and Mutlu (2001) reported in the activation energy of biochemical reaction catalyzed by free and immobilized commercial pectinase was 9.4 and 11.9 kcal mol^{-1}, respectively. The activities of free and immobilized enzymes for various substrate concentrations are plotted in Lineweaver–Burk graph, from which maximal activities (V_{max}) and Michaelis–Menten constants (K_m) values were calculated (Fig. 3). The K_m was shifted from 2 to 3.2 mg ml^{-1} free and immobilized enzyme respectively. Also, the V_{max} of the free enzyme decreased from 5 to 3.8 mg ml^{-1} min^{-1}. In this concern, the Kinetic constants V_{max} and K_m were 0.005% (w/v)/s and 1.137% (w/v) pectin, respectively for free enzyme and 0.009% (w/v)/s and 2.172% (w/v) pectin, respectively for immobilized pectinase (Sarioglu et al., 2001).

3.6. Orange juice clarification

Clarification of fruit juices applying low cost and good quality is an important goal in the industrial application. Accordingly, the

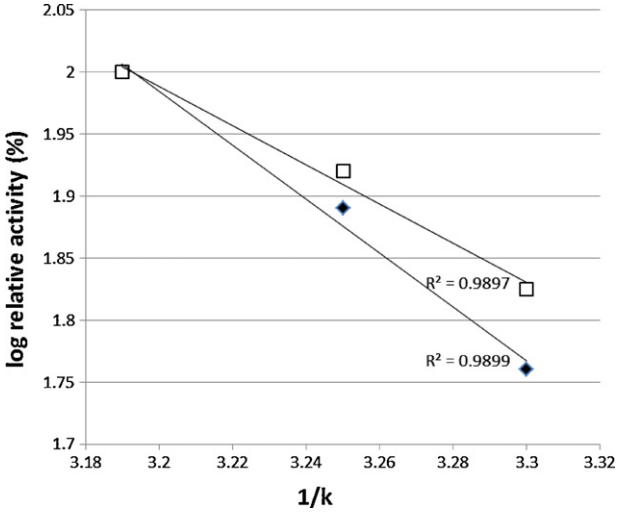

Fig. 2. Arrhenius plots for the free and immobilized pectinase.

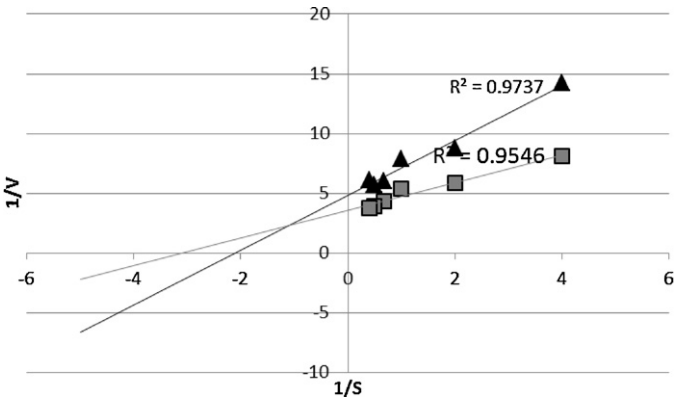

Fig. 3. Lineweaver–Burk plot, of free and immobilized pectinase from *Aspergillus niger NRC1ami24* for citrus pectin.

following trials in this study were carried out to evaluate the free and immobilized *A. niger* NRC1ami pectinase for orange juice clarification. The treatment of the orange juice with the immobilized pectinase afforded clear orange juice while many haze particles remained in juice treated with the free enzyme form (Fig. 4). It was reported that, in the presence of calcium ions, insoluble calcium pectate is formed in orange juice as haze particles (Kashyap et al., 2001). The disappearance of haze in case of the immobilized enzyme predicted that the pH shift in the immobilization process toward the alkaline range activated the pectin lyase and suppressed both the polygalacturonase which represented the main enzyme in this study and also the pectin methyl esterase. Kobayashi, Koike, Yoshimatsu, Higaki, and Suzumatsu (1999) reported in highly active-pectate lyase from an alkaliphilic strain of *Bacillus* sp. In this study the pectin methyl-esterase enzyme was detected according

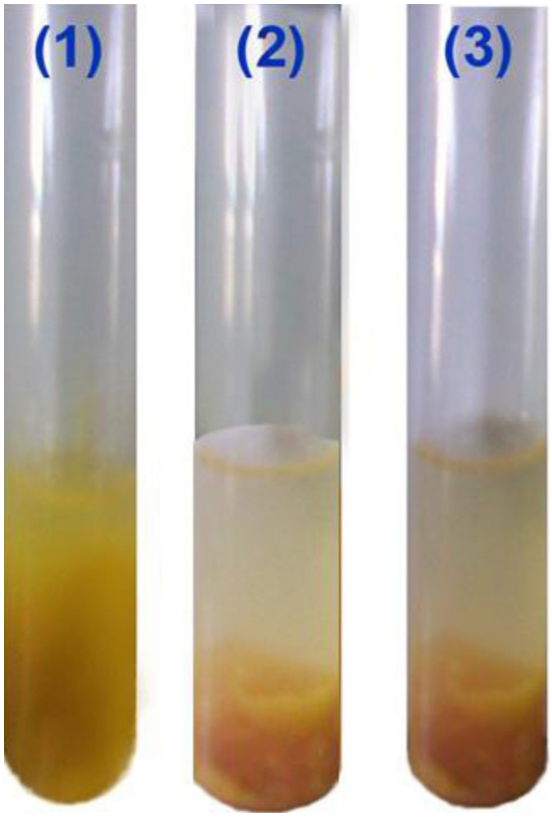

Fig. 4. A simple trial to evaluate the efficiency of the free (2) and immobilized enzymes in orange juice clarification (3), control juice without enzyme (1).

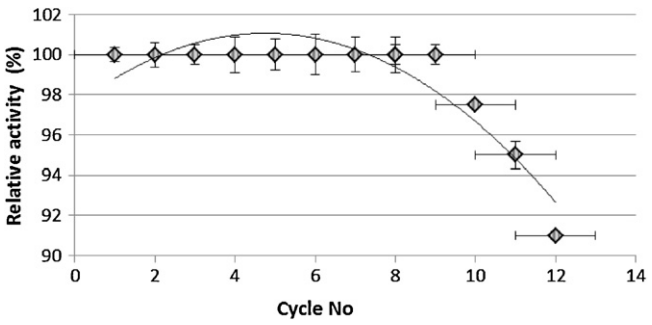

Fig. 5. Re-usability of the immobilized *Aspergillus niger NRC1ami24* pectinase using orange juice as substrate.

to the reduction in the orange juice pH from 3.5 to 2 and 3.5 to 3.1 when the free and immobilized enzymes were used, respectively. From this result it was concluded that the immobilization process played a role in reduction of pectin methyl-esterase activity and methanol release. The reduction percent in orange juice viscosity with the free and immobilized enzymes were 69%, 75% respectively. All the previous results insure that the clarification process using the immobilized pectinase was considered efficient and promising due to the great reduction of haze and viscosity, and by showing no significant changes in pH. Similar results were reported in clarification of orange juice (Carneiroa, Saa, Gomesb, Mattab, & Cabralb, 2002).

The operational stability of the immobilized and free enzyme was tested in the presence of orange juice as substrate (Fig. 5). The immobilized enzyme was successfully reused 12 times in orange juice clarification with the activity loss 2.5% and 9% at 10 and 12 cycles respectively. This value was better than that reported by Ipsita, Meryam, and Munishwar (2003) for alginate entrapped pectinase which exhibited 55% loss in activity after repeating the reactions 4 times. On the other hand, Li et al. (2008), reported that 81% residual activity was observed in the immobilized enzyme after 10 batch reactions using haw pectin as substrate.

4. Conclusion

This study aimed to the evaluation of *A. niger* NRC1ami pectinase for some industrial applications. The untreated (UOP) and treated (NOP and HOP) orange peels afforded considerable degree of saccharification. All the properties studies of the immobilized enzyme in polyvinyl alcohol exhibited its superiority compared to the free form. Furthermore, the immobilized enzyme showed efficiency in orange juice clarification, which was better than the free form. Also all the kinetic properties referred to the changes in the enzyme molecule. The previous results insure the primacy of the immobilized *A. niger* NRC1ami pectinase compared to many other pectinases.

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