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Immobilization of Invertase by a New Economical Method Using Wood Sawdust Waste

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Abstract: A novel technique was adopted using wood waste as a new carrier for adsorption of invertase. The new method improved activity, pH and thermal stability of the immobilized invertase from *Saccharomyces cerevisiae* and was more resistant to washing out by concentrated salt solution (6M NaCl). This result encourages using sawdust as matrix for purification of invertase or may be for other enzymes. Optimum conditions for activity were not affected by immobilization and it was found that the optimum pH and temperature for either free or immobilized enzymes were 5.6 and 60 ^{0}C respectively. Immobilized invertase was however, more stable at high pH and temperatures. There was no leaching of the enzyme for at least 2 months when stored in refrigerator. The immobilized invertase was less sensitive to inhibition by impurities found in molasses and was applied for continuous sucrose hydrolysis in column bioreactor. This method can be used for the industrial production of invert sugar.

Key words: *Saccharomyces cerevisiae*, invertase, immobilization, wood shavings and sawdust.

INTRODUCTION

Invertases are special kind of enzymes that catalyze the hydrolysis of sucrose into glucose and fructose. Invert sugar syrups are mainly used in confectionary, beverage industries and bakeries. Immobilized yeast invertase has been the major enzyme used for commercial production of invert sugar (Basha and Palanivelu, 2000). Physically adsorbing an enzyme onto a matrix is the oldest immobilization technique. It is characterized by its simplicity and by the fact that undesirable chemical effects are avoided, since adsorption is usually a gentle procedure. By this sort of immobilization neither the enzyme activity changes nor catalytic characteristics of the enzyme (Woodward, 1985). Possible supports for the adsorptive or ionic binding of enzymes are for example anionic or cationic resins, active charcoal, silica gel, clay, aluminum oxide and porous glass (Woodward, 1985; Walcarius, 1998 and Tomotani and Vitolo, 2006). Natural cellulose deserved special attention for characteristics as high adsorptive capability. It is found in cotton fibers and wood. Wood is a material source for energy and constructional works but byproduct of wood processing, pollutes the environment. Wood chips, slabs, shavings and sawdust account for the half of the total wood waste of wood processing. The study will prove that wood waste could be a product looking for a market. From the separation science point of view sawdust can be considered as a chromatography material or adsorbent (Safarik *et al*., 2005). It has been shown recently that sawdust has affinity for different biologically active compounds such as enzymes (palonen *et al*., 2004). Also different organic compounds such as acid and basic dyes and oils (Shukla *et al.,* 2002 and Garg *et al*., 2004) and heavy metal ions (Yu *et al*., 2001) were efficiently adsorbed on different types of sawdust. There is little information on using wood waste as a carrier for enzyme immobilization. Jin and Toda (1988) immobilized papain (E C.3.3.3.10) by covalent binding to wood sawdust. Munish *et al.* (2005) immobilized Naringinase (E C.3.2.1.40) by covalent binding to wood chips. Although invertase has been immobilized over the last 90 years in uncountable types of supports through all kinds of immobilization techniques, the idea of immobilizing invertase by adsorption on wood sawdust has been hardly ever reported in the literature.

This work presents a new and simple method for immobilization of invertase onto wood shavings or sawdust by adhesion. This immobilization technique was done by autoclaving wood shavings or sawdust before adding the enzyme in order to expose the support to high pressure as a kind of activation. The study will discuss the potential for wood waste-invertase application to the industrial hydrolysis of high concentrated sucrose solutions.

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MATERIALS AND METHODS

Chemicals:

Sucrose was obtained from BDH, Sugarcane molasses from Sugar Company of integrated industries, Egypt.Sugar beet molasses from Kafr El-Sheikh Sugar Company, Egypt. All the other chemicals used were obtained from Merck.

Enzyme:

Invertase was obtained from baker's yeast by mechanical techniques (shaking with glass beads), centrifuged then concentrated with ethanol.

Assay of Invertase:

The soluble invertase was assayed in 1 ml reaction volume containing 0.2 ml enzyme and 0.8 ml of 2.5 % sucrose in 0.1 M sodium acetate buffer (pH 4.8). The immobilized invertase was assayed in 1 ml reaction volume containing 0.1 g bound enzyme and 1 ml of 2.5 % sucrose in the same buffer. The amount of reducing sugars in the supernatant was estimated with dinitrosalycylic acid.

Immobilization of Invertase on Different Carriers:

Immobilization of invertase was carried out by adsorption on different carriers to compare their adsorptive capability with that of wood sawdust. The carriers used were: pumice, lufa, kaolin, foam, sponge, active carbon, Alumina, Amberlite resins IRC-50, acidic Dowex, basic Dowex, chitosan, Silica gel, filter paper, ecteulla cellulose, wood sawdust and natural wool. To 0.5 g of the above mentioned carrier, 2 ml of enzyme (400 unit/ ml) was added and soaked for 2 - 4 hrs. at 30^{o}C with gentle agitation. After fixation (immobilization), the material was washed several times with assay buffer; this ensures that non-adsorbed enzyme is removed.

Immobilization of Invertase on Wood Sawdust:

Sawdust was autoclaved at 1.5 atmospheric pressures for 15 min without any treatment. To each 0.5 g of sterile or non-sterile sawdust, 2 ml of enzyme (400Unit/ml) was added and soaked for 2 - 4 hrs. at 30^{0}C with gentle agitation, then wash several times. The same procedure was followed with the other type of wood waste. The efficiency of enzyme immobilization was evaluated by different parameters including the retained enzyme activity, the specific activity of the immobilized enzyme and the loading efficiency. The immobilization yield is the key parameter, since it represents the general output of the efficiency of the immobilization process.

Continuous Hydrolysis of Sucrose Using Column Reactor:

The enzymatic hydrolysis was carried out in continuous system. 0.25 L column reactor of 2.5 cm inner diameter and height 45 cm was used. The reactor was packed with 5 g wood shavings loaded with (3950 units / g carrier). The substrate solution was 5% sucrose and was fed using peristaltic pump with rate of 5 ml / min.

Protein Determination:

This was done by the method of Lowry *et al*. (1951).

RESULTS AND DISCUSSIONS

Preparation of Invertase Enzyme:

Yeast and especially *Saccharomyces cerevisiae* has shown great ability to secrete invertase (Moreno *et al*., 1979; Silveira *et al*., 2000 and Shafiq *et al*., 2002). Therefore, baker's yeast was chosen as source of invertase in this study. Invertase obtained from baker yeast in soluble form where cells were broken down by mechanical shaking with glass beads followed by differential centrifugation of the cell extract, indicating lack of covalent linkages between invertase and cell wall structural components (Rodricuez *et al*., 1995).

Comparison of the Adsorptive Capability of Different Matrixes to Invertase:

Among all types of adsorbing materials used for this experiment, it was found that each of foam, active carbon and acidic exchange resin (Dowex) failed to retain any invertase activity. It was expected that the supports such as lufa, sponge, wool, gave high immobilization yield since they are characterized by high porosity but unfortunately their immobilization yield were 10, 25 and 30 % respectively. This is due to the

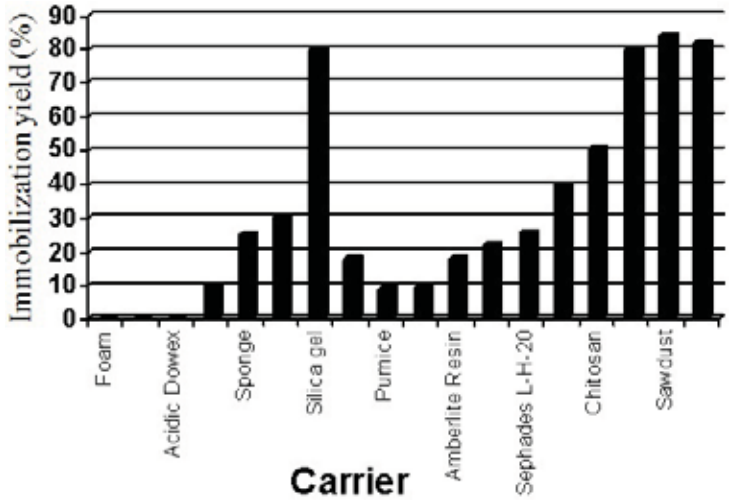

Fig. 1: Comparison of the adsorptive capability of different matrixes to invertase

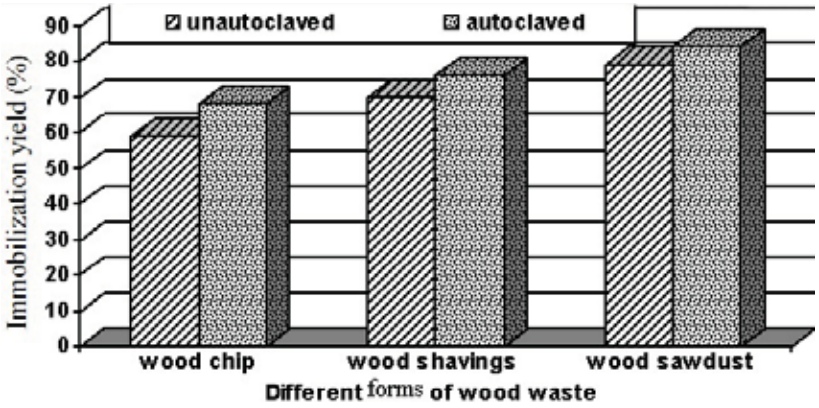

Fig. 2: Immobilization of invertase on different forms of wood waste

week binding between the enzyme and the support. However, Krastanov (1997) reported that yeast cells adsorbed well on wool using glutaraldehyde. Souza and Kamath (1988) obtained the same result on cotton using polyethylenmine. Although using such binding agents increase the efficiency of immobilization but the present work aimed to study the natural adsorption property.

In contrast to kaolin, silica gel gave considerably high immobilization yield 80 %. Silica gel is a porous, granular form of silica, its surface have hydroxyl groups which are responsible for adsorption properties (Jal, 2004).The structure of kaolin is hydrated aluminum silicate (Ahmed 2005), this structure may be responsible for the lower immobilization yield (18 %).

Fig. (1) shows that, pumice, Alumina, Amberlite, DEAE-Sephadex 50, Sephadex L-H-20 and filter paper gave immobilization yields 9, 9.5, 18, 22, 25.5, 40 % respectively. The variation of immobilization yields from 9 to 40 indicates that the nature of the matrix determines its physical properties such as its behaviors towards enzyme and, to a certain extent, its capacity.

Although most of invertase (in un-shown data) applied to chitosan matrix was adsorbed on its surface, but its immobilization yield was 50%. This indicates that invertase may bind to chitosan via protein moiety which affects the active site of invertase. Husain *et al.* (1996) reported that invertase is a glycoprotein enzyme and it can be immobilized onto chitosan via either its carbohydrate moiety or protein moiety. The use of carbohydrate moiety for linkage to solid supports generally does not affect the active site of invertase, since it is located to the protein moiety.

Cellulosic materials have been widely used as carriers for immobilized enzymes due to their hydrophilic character and the great number of hydroxyl groups on the surface capable of chemical reaction. It is known that matrixes such as ecteola cellulose, and wood sawdust gave the highest immobilization yield 80, 84 % respectively.

Fig. (1) also shows that basic anionic exchange resin (Dowex-1x 4-200) gave high immobilization yield (82%). These distinct adsorptive capabilities are due to that Dowex-1x-4-200 constituted by 2% of divenylbenzene (8% of styrene and beads of 400 mesh, leading to high superficial area. (Li *et al.*, 2001).

It is interesting to note that invertase bound to the matrix which contain hydroxyl group. Matrixes such as silica gel, ecteola cellulose and Dowex IR (40 - 200 mesh) are good matrixes but sawdust is the cheapest one.

Immobilization of Invertase on Different Forms of Wood Waste:

Invertase was immobilized on wood chips, shavings and sawdust to investigate the influence of support size on the relative activity. It was observed that wood sawdust adsorbed highest amount of invertase compared to the other forms of wood waste. These distinct adsorptive capabilities undoubtedly linked to the superficial area available for the enzyme molecules. The activity of wood-bound invertase increase when the amount of bound enzyme is increased due to increase wood surface area.

As shown in Fig. (2), autoclaved wood waste gave higher activity than the non-autoclaved one. The idea of autoclaving is based on that wood is composed of lignified cellulose and exposure of wood waste to such high pressure of autoclaving (1.5 atmospheric pressure) may be responsible for physically partial removal of lignin leading to increases of cellulosic area free of lignin (hydrated cellulose). This hydration process of cellulose may be responsible for more adhesion of invertase. In all experiments sawdust was used as a support except in scanning electron microscopy and packing column reactor.

Scanning Electron Microscopy:

Microscopic photograph of adhered invertase is shown in Figs. (3 & 4). One piece of either autoclaved or un-autoclaved wood shavings about 2 mm x 2 mm x 0.05 mm loaded with invertase placed on a scanning electron microscope sample holder and coated with gold by using an E. microscope sputter coater (model ES 500). The gold-coated samples were viewed with a Philips electron microscope (model XL 30-FEG). Sawdust was substituted by wood shavings to increase the clarity of the picture.

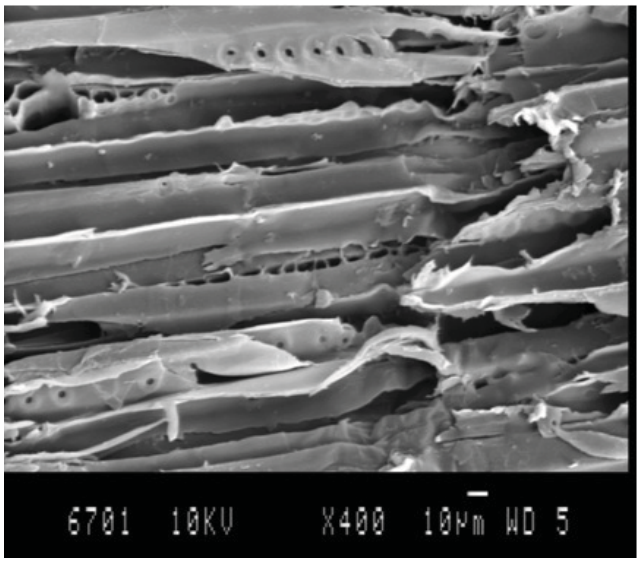

Fig. 3: Invertase on autoclaved surface.

A transparent white thin film on the un-autoclaved support formed by invertase (Fig. 4) can be seen. As shown in Fig. (3), high pressure of autoclaving affect the wood fiber that it looses the fibers allowing for more adhesion of invertase between the fibers.

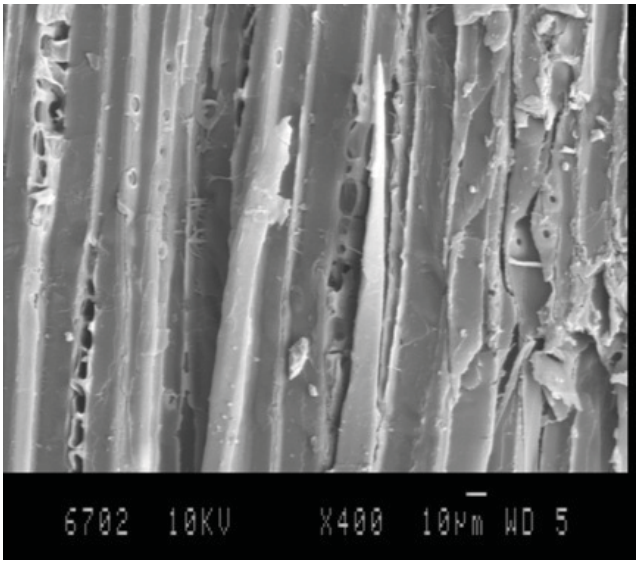

Fig. 4: Invertase on un-autoclaved surface.

In un-shown data, the stability of the immobilized invertase was determined by subjecting the autoclaved and un-autoclaved supports to overnight washing in sodium acetate buffer (buffer of assay). While none of the immobilized invertase was removed after 24 hr. from autoclaved wood sawdust, while more than 40 % of the bound invertase was removed from the un-autoclaved surface. For this reason autoclaved wood sawdust better for immobilization of invertase than using un-autoclaved one, although no appreciable difference in immobilization yield of both.

Stability of Adsorbed Invertase on Sawdust:

The novel immobilization process of invertase on autoclaved sawdust led to remarkable stability (thermal, pH or storage stability) of the enzyme as a compared to the free form or as compared to other methods of immobilization for the same enzyme from different sources.

Prodanovic[ŷ] *et al*. (2003) reported that adsorbed periodate oxidized invertase on sepiolite retained 91.6 % of its initial activity after rinsing with 1M NaCl for 4 days at 4^0C and referred that to the formation of oligomers of enzyme molecules on the periodate oxidized invertase. Xiaohua *et al.* (1994) reported that adsorbed invertase on alumina by cross-linking with glutaraldehyde are difficult to wash out because of the chelate effect.

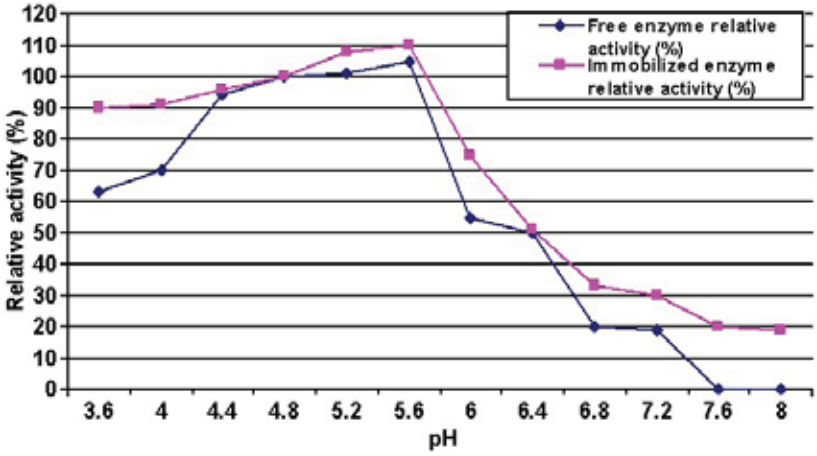

Fig. 5: Effect of pH on relative activity of free and immobilized invertase.

Adsorbed invertase on autoclaved sawdust retained 97 % of its initial activity after rinsing with 6 M NaCl for more than one week, although the mechanism of this strong binding is up till now to some extant not clear, this result prompts using sawdust as matrix for purification of invertase in further study. Adsorbed invertase on sawdust protected the enzyme in acidic and alkaline pH. As shown in Fig. (5), immobilized invertase was more stable than free enzyme at pH 7.6 and 8.0. Free enzyme was completely inactivated at high pH. The optimum pH was 5.6 for both forms of enzyme. For cross-linked yeast invertase, optimum pH was 6.0 and free enzyme 6.5 (Kaplan and Bakir, 1998). The same result was reported for *Thermomyces* invertase on phenyl-sepharose (Basha and Palanivelu, 2000).

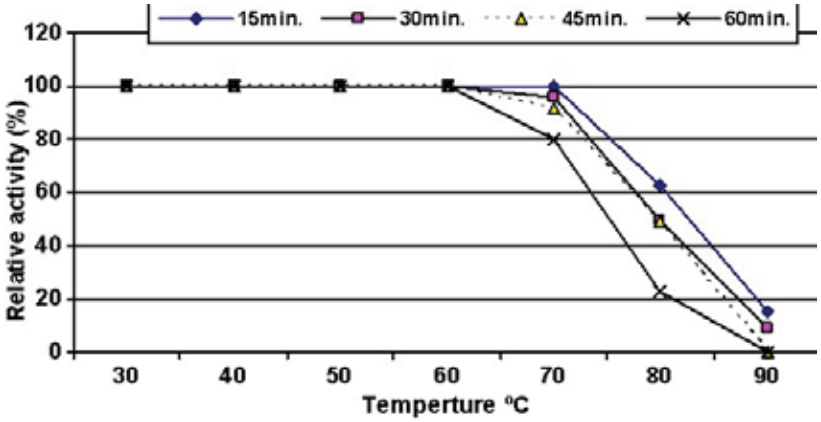

Fig. 6: Thermal stability of immobilized invertase after treatment of enzyme at different temperatures at different time intervals.

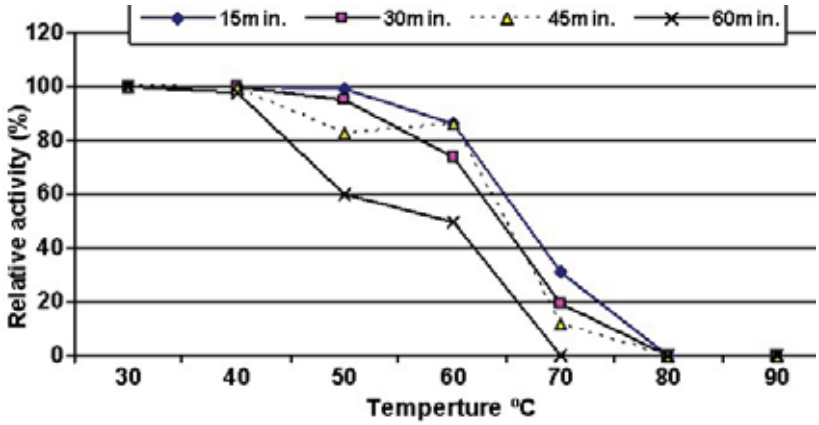

Fig. 7: Thermal stability of free invertase after treatment of enzyme at different temperatures at different time intervals.

Regarding thermal stability, the immobilized invertase showed remarkable stability as compared to the free invertase (Figs. 6 & 7). The immobilized invertase retained 100 % of the activity after 1 hr. at 60 ^{0}C, whereas the free invertase retained only 50 %. However, optimum temperature for both is 60 ^{0}C and this indicates that free enzyme is more stable in presence of its substrate. Free invertase was completely inactivated at 80 and 90 ^{0}C, meanwhile immobilized enzyme retained 63 % and 15 % of its original activity after 15 min at the same previously mentioned temperatures respectively.

Tanriseven and Dôgan (2001) obtained approximately the same results when immobilized invertase within calcium alginate gel capsules and their immobilization technique kept invertase for 36 days without decrease in activity where sawdust kept invertase for 60 days also without loss in activity. While immobilized invertase on carboxymethylcellulose modified chitin kept invertase for 50 days of storage after loss 5 % of its activity (Gòmez *et al.,* 2006).

Effect of Different Concentrations of Substrate on Invertase:

The effect of substrate concentration on the free and immobilized invertase was studied using different concentrations of sucrose as the substrate ranging from 10 mM to 1000 mM. Fig. (8) indicates that a parallel relationship existed between substrate concentration and enzyme activity up to 200mM and 400 mM for free and immobilized invertase, respectively. Higher substrate concentrations resulted in a constant enzyme activity for immobilized enzyme and little inhibitory effect on free enzyme.

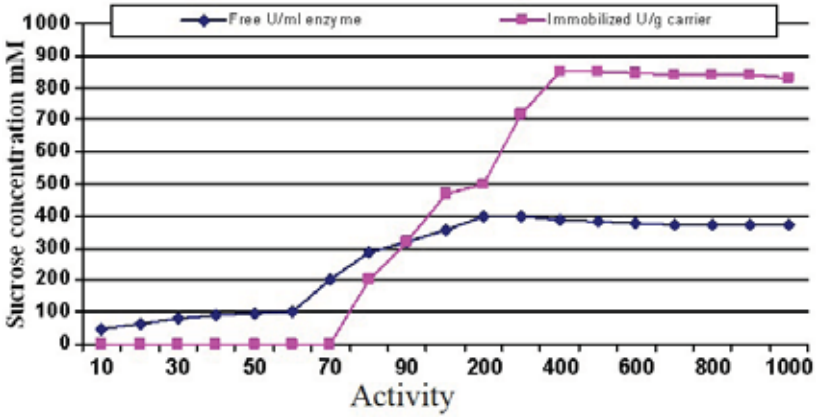

Fig. 8: Effect of different concentrations of substrate.

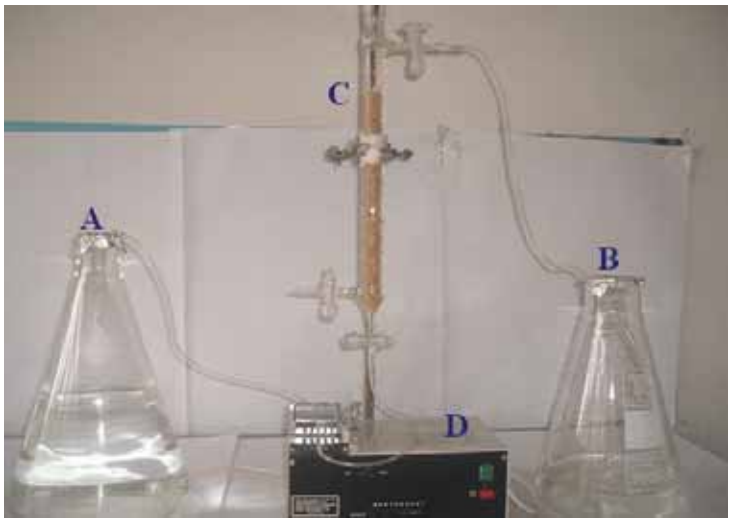

Fig. 9: Column bioreactor (A): Container of substrate, (B): Container of product, (C): Bioreactor filled with carrier, (D): Peristaltic pump.

Immobilized invertase need higher sucrose concentration than free enzyme and this may be due to, lower substrate concentrations are not enough to reach to the active site of enzyme inside the support. In un shown data, substitution of sucrose with other types of substrate such as sugarcane and sugar beet molasses proved that immobilization process protected the enzyme from impurities of molasses which inhibited free invertase. The overall performance of immobilized invertase on wood waste is rather promising than the free enzyme.
Continuous hydrolysis of sucrose and operational stability

Because the flow properties of shavings is better than fine sawdust in such column bioreactor, shavings was used. But sawdust can be used in another type of bioreactors.

Immobilized invertase on wood shavings retained 90 % of the original activity after 20 cycles of reuse in batch reactor (5 hrs, each cycle = 15 min.) and it retained 65 % of the original activity after 10 hrs. of continuous operational regime in a column reactor with flow rate of 300 ml /hr./5g carrier.

Although using wood waste as a carrier for invertase did not offer long life immobilization system but the high productivity of this system considered as an advantage superior to other immobilization systems in the literatures. It was found that 3000 ml of 5% sucrose solution was converted to fructose and glucose solution using 5 g carrier only, i.e., 100 g wood waste can convert 3 kg sucrose. The results obtained from this study lead to the suggestion that a further scaling up is possible especially at high concentrated sucrose solutions while adjusting flow rate can increase operational stability.

Wood Waste Can Become a Product Looking for a Market:

Since economical disposal of wood sawdust, shavings remain a problem of growing concern to the wood industry. Major emphasis in this work was placed upon established or developing uses rather than upon potential uses. In this respect this direction of research may be of interest in the innovative use of wood waste as a carrier for enzyme immobilization and purification. Even though sawdust have been recently used to immobilize very few enzymes (rather than invertase) this procedure is a novel one for immobilization of invertase and other enzymes because no information is available on immobilization of any enzyme on sawdust by natural adsorption or by exposure of sawdust for high pressure as a step of immobilization.

The economic feasibility of the process is directly related to the costs of the reagent. This investigation indicates that exposure of wood waste to high pressure (autoclaving) as a kind of activation of support for invertase immobilization process is safe and of economic interest, especially in the application of continuous hydrolysis of sucrose on pilot or industrial scale. In comparison with the method of Mansfeld *et al.* (1992) or Gomez *et al.* (2006), it was found that the new method is more economic, safe and efficient. The production of pure invert sugar from waste (molasses) using wood waste (sawdust or shavings) under harsh conditions (high temperature & high pH) is attractive to industrial application.

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