



Eco-Friendly Technique for Invertase Immobilization and Oligosaccharide Synthesis at Low Temperature

Doaa A.R. Mahmoud

National Research Centre, Department of Chemistry of Natural and Microbial Products Dokki, Giza, Egypt.



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ECO-FRIENDLY technique for invertase immobilization has been naturally created in the present study. It is remarkably simple and does not need extensive preparation of a highly pure enzyme or an expensive carrier. The used method relies on mixing pretreated sawdust with baker's yeast in a closed system and comparing it with that in an open one. The technique properly introduces a practical and novel insight into the role of carbon dioxide in possible modification of sawdust's surface and invertase immobilization. It additionally provides vital insights on the key role of immobilization technique on the excellent stability and activity of the enzyme. The storage stability of the immobilized invertase retained 93% of its activity after keeping of 2 consecutive years at 4 °C. Although invertase had been largely immobilized on different cellulosic materials, but the novelty of the present study represent the enzymatic synthesis of oligosaccharide by cold-adapted invertase at 20 °C.

Keywords: Oligosaccharide; Invertase; *Saccharomyces*; Enzymatic synthesis; Sawdust; Cold active.

Introduction

In the context of the global need for sustainability and natural manufacturing technologies, biocatalysts are a great alternative to chemical transformations [1 & 2]. The application of cold-adapted enzyme offers numerous advantages such as catalytic efficiencies in the temperature range of 0-20°C prevent the risk of microbial contamination especially in continuous systems expensive heating/cooling systems thus constituting a considerable progress towards the saving of energy.

Surprisingly only little old literatures have been examined production of cold active invertase [3,4,5]. Invertase (EC 3.2.1.26) is hydrolyzing enzyme called beta-fructofuranosidase. It cleaves the terminal non-reducing beta-fructofuranoside residues. It is one of the most studied enzymes due to its extensive industrial applications. It is inexpensive and can be easily obtained. Invertase

has a footprint in the sugar market, because it is utilized in the beverage, baking and confectionery industries [6]. Because of enzyme availability, there is no practical interest in its improvement by mutations [7]. However, further improvement of enzyme stability justifies the necessity of immobilization. Immobilized yeast invertase has been the major enzyme used for commercial production of invert sugar [8].

For application in industry the cost contribution of the (immobilized) enzyme is an important issue. Clearly, the immobilization methodology, in addition to providing an active and stable biocatalyst, should be a relatively simple operation that does not require a highly pure enzyme preparation or an expensive support that may not be commercially available [9]. Immobilization on cellulosic wastes especially sawdust typically meets all these necessary criteria which indicate they have promising

*Corresponding author e-mail: doaanrc1993@yahoo.com

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industrial potential due to their high activity retention and stability in addition to ease of preparation from crude enzyme samples.

Oligosaccharides are known as “functional food ingredients” because they have human health benefits. Fructooligosaccharides are food bioactive carbohydrates which do not hydrolyze by digestive enzymes of intestine or saliva. They can reach the colon without any change. Intestinal bacteria fermented these oligosaccharides, forming short-chain fatty acids which are important to renew and maintain lining cell of large intestine. Moreover, they stimulate immune system and prevent the growth of harmful bacteria [10]. Indispensable for the use of enzymes in various areas of daily life especially the immobilized one therefore, to increase competitiveness of immobilized enzyme for technical applications their costs must be reduced [11]. Innovative technique has been created to immobilize invertase in presence of CO₂ in closed system. Through many readings in the scientific literatures about all types of enzyme immobilization or of all techniques for the generation of functional immobilized enzymes, no one mentions the utilization of sawdust as a carrier by this technique. Therefore in present study potential of saw dust for invertase immobilization and oligosaccharides synthesis at low temperature were discussed. Based on the economic importance of invertase enzyme in industrial applications, interest in improving its performance for oligosaccharides production is required.

Materials and Methods

Materials

Sucrose was obtained from BDH, acetic acid from El Naser Company, Sodium acetate and all the other chemicals used were obtained from Merck. The commercial live bakers' yeast, *Saccharomyces cerevisiae* was obtained, in two forms, one form of active dry yeast, from the Egyptian company for advanced Foodstuff Industries. The fresh compressed yeast brought from Sugar Company of integrated industries, Egypt. Baker's yeast used without further purification. Sawdust samples were obtained from local sawmills.

Methods

Preparation of invertase

Different weights of dry or compressed yeast were suspended in 100 ml of distilled water and the mixture stirred for 2 min. Yeast external invertase released gradually in the solution [12].

Auto-immobilization technique

Pretreatment of sawdust was conducted by autoclaving it for 15 min. at 121°C. The previous solution was incubated with pretreated sawdust at temperature 35 °C in a rotary shaker for range of 0.5-2 h. At the end of the incubation period, the sawdust washed. This process was repeated several times to ensure the complete removal of yeast cells, and the supernatant was discarded. The washed sawdust was considered as treated sawdust. Yeast external invertase that has been released in the solution, immobilized automatically on the sawdust.

Assay of invertase

Invertase enzyme assay was conducted according to the modified method of [13]. The reaction mixture contained 0.1 g bound enzyme (invertase on sawdust) and 2 ml of 100 m Mole sucrose in sodium acetate buffer (0.1 M & pH 5.2) at 50°C for 15 min. The amount of reducing sugars in the supernatant was estimated with Somogyi reagent.

Oligosaccharide synthesis

The immobilized invertase (0.1g) was mixed with 2 ml of 100 m Mole sucrose in sodium acetate buffer (0.1 M & pH 5.2) at 20°C for 2 hr up to 12 hr. At the end of the reaction time, the supernatant was spotted on chromatogram paper Whatman No.1 using the solvent mixture n-butanol: acetone: water (4: 5: 1, v/v/v).

Sawdust characterization by SEM

The morphology of treated, pretreated and untreated sawdust was investigated by scanning electron microscope (SEM) model Quanta 250 FEG (Scanning Electron microscope).

Sawdust characterization by (FT-IR)

Fourier transform infrared spectroscopy (JASCO FT-IR 6100, made in Japan). FT-IR was conducted to analyze the structural and chemical changes of sawdust due to the different treatments. At ambient temperature, the wavelength region from 4000 to 500 cm⁻¹ were taken.

Measurement of CO₂

Sample of 20 cm³ CO₂ was withdrawn with syringe through opening above the bottle cap. The concentration of CO₂ was measured using Testo 353 sensor according to the instrument instruction. First, the value of CO₂ was adjusted to the nominal value and stored. Then calibration/adjustment carried out. Before completing the adjustment, the instrument left for 3-4 min., displayed the current CO₂ measuring values.

The values were zeroed as a reference. Then hold down button was pressed until the display switched to the measurement values. The readings of the measuring values were taken. The reading was expressed in parts per million (volume/volume). Each reading represented the amount of CO_2 in 1 cm^3 .

Results and Discussion

Mechanism of invertase immobilization

To understand the mechanism by which invertase was immobilized on sawdust, the author put assumption and proved it.

The assumption was: by incubating baker's yeast with sawdust at certain temperature for a period of time under shaking condition, CO_2 supposed to be liberated and invertase secreted. The CO_2 produced from this reaction employed as a reactant and modified the cellulosic surface of the sawdust. This technique was not based on the adsorption of CO_2 on the sawdust surface but CO_2 involved as a reactant. Therefore, we can anticipate that this technique might increase the capacity of invertase that had been secreted from baker's yeast to be immobilized on the cellulosic surface of sawdust. **Figure 1** illustrated this

mechanism. It is important to point out that this method can be applied on any cellulosic wastes but wood wastes have been selected as a model example [14].

The role of baker's yeast and CO_2 in invertase immobilization

Yeast is the source of invertase and also the source of CO_2 . By mixing baker's yeast with sawdust in presence of traces of sucrose, it behaved in different ways. First yeast adjusted to the new environment and began to grow in size and entered initial lag phase. Then yeast entered the exponential growth phase, where yeast began to reproduce increasing their number exponentially, carbon dioxide released in large quantities [15]. To prove that CO_2 induced invertase immobilization and to confirm that any changes in sawdust surface were as the results of CO_2 bubbles in the fermentation medium. The incubation of baker's yeast with sawdust was conducted in two different systems. One system is opened as shown in **Figure 2** and the other is closed as shown in **Figure 3**. The liberated CO_2 captured in lime water as shown in **Figure 2**. In the closed system, CO_2 accumulated in different concentrations according to the reaction conditions, causing pressure on sawdust.

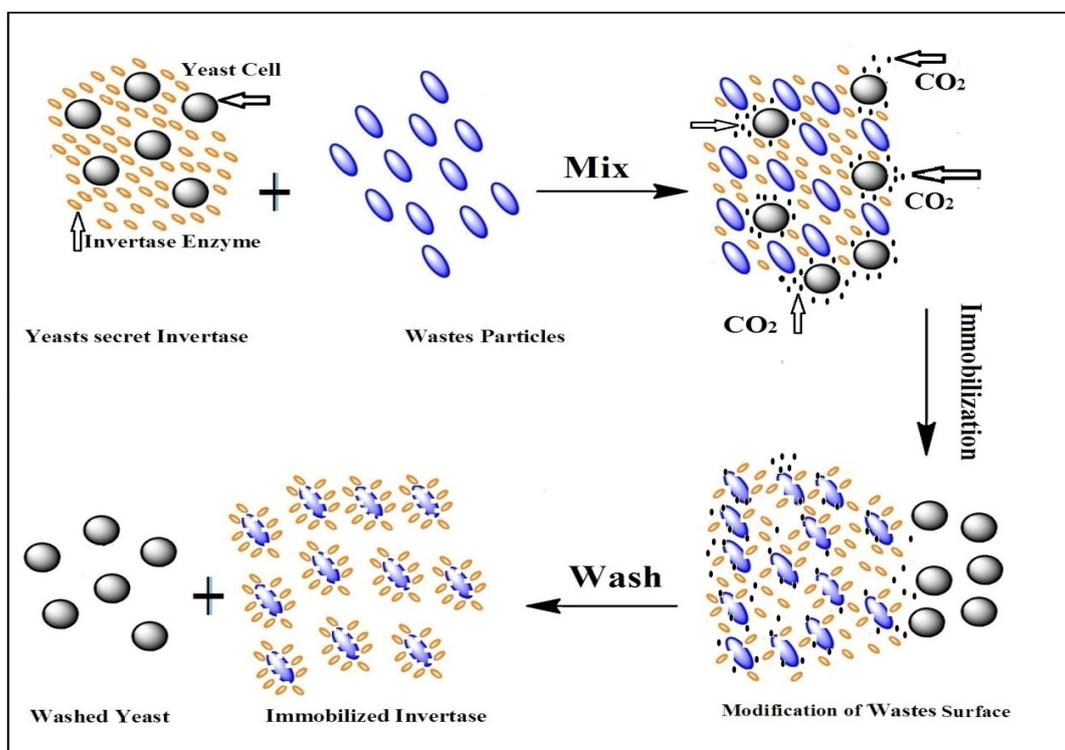


Fig. 1. Author illustration for invertase immobilization on sawdust

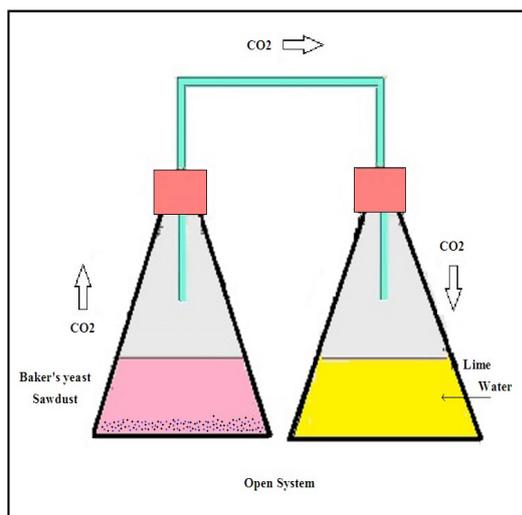


Fig. 2. Immobilization process in opened system.

The experiments were conducted in screw bottles of 200 ml volume. Each bottle contains 1g pretreated sawdust and 1g baker's yeast in 50 ml H_2O . In the closed system, at the top of the bottle cap, there is a tightly closed aperture. A syringe is inserted to measure the amount of CO_2 in that aperture. It is important to mention that; invertase secreted by baker's yeast was immobilized automatically on sawdust. Unfortunately, there is no method to measure the amount of immobilized invertase, but the evidence for its existence is the measure of its activity. At the end of the experiment, sawdust from both systems was washed thoroughly with water, and this step repeated several times. 0.1g of sawdust was weighed as an immobilized invertase and its activity was measured as previously mentioned.

The results in **Table 1** indicated that invertase activity was 22.5 U/ g carrier in open system. While invertase activity in closed system was 76.5 U/ g carrier, which meant it was 3.4 fold greater. The explanation for this result was that; the liberated CO_2 modified the sawdust and hence increased the binding sites for invertase [16]. As mentioned before sawdust was pretreated by autoclaving, which assisted in partial removal of lignin [17]. This step facilitated the binding of invertase in open system. The liberated CO_2 in the closed system was trapped. As such, the level of CO_2 pressure in the bottle increased. In turn, free cellulose increased due to disruption of the cellulosic structure of sawdust [18]. This step facilitated the binding of more invertase. This will be confirmed later as yeast concentrations and incubation periods change.

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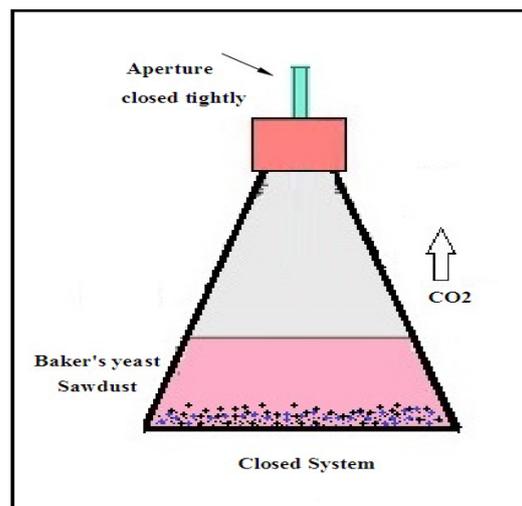


Fig. 3. Immobilization process in closed system.

Morphological characterization of saw dust

It is reasonable to expect that CO_2 made the surface of sawdust more degraded and it disrupted the structure of cellulosic materials; thereby increasing the amount of free cellulose available for binding with the enzyme [19].

The morphology of sawdust was investigated by scanning electron microscope as shown in **Figure 4**. Three different samples of sawdust were investigated. The first sample was untreated sawdust (micrograph A) the fibers showed an integrated structure. The second sample was the pretreated sawdust (micrograph B) surface was partially fibrillated. The third sample was the baker's yeast treated sawdust (micrograph C) fibers showed a ruptured cell wall structure. After treatment, sawdust samples showed a more disrupted structure.

Effect of different incubation time on invertase immobilization

To demonstrate that increased CO_2 concentration stimulated invertase immobilization and activity; the effect of different incubation time was investigated. The time was adjusted between 0.5 and 2 h. The amount of CO_2 was measured quantitatively using a CO_2 gas sensor (Testo Model 353).

The results **Figure 5** indicated that an excessive increase in carbon dioxide amount by time facilitated the faster penetration of carbon dioxide molecules on cellulosic surface of sawdust. The longer the incubation of sawdust with yeast, the greater the amount of CO_2 released, the greater the immobilized enzyme.

The cellulosic structure of sawdust was severely disrupted by the effect of CO₂. This disrupter increased the accessible surface area of for more immobilized invertase. Hence yeast external invertase is a glycoprotein, and then it was easier for invertase to bind by its glycosylated bonds to the free cellulose of the treated sawdust [20]. After 2 h the activity was 91.7U/g carrier at CO₂ 129 ppm. On contrary, increasing the time

above 2 hours yielded more CO₂ but no more invertase activity. Significant invertase activity decreased. The author attributed this result to the fact that sawdust reached to full saturation with invertase. At concentration 135 ppm, invertase started to denature [21]. The conclusion from this experiment is that incubation should be long enough to permit a moderate amount of product to be formed [22].

TABLE 1. Effect of CO₂ & baker’s yeast on invertase immobilization.

Baker’s yeast	Auto immobilized invertase in closed system	Auto immobilized invertase in opened system
Invertase activity U / g carrier	76.5	22.5

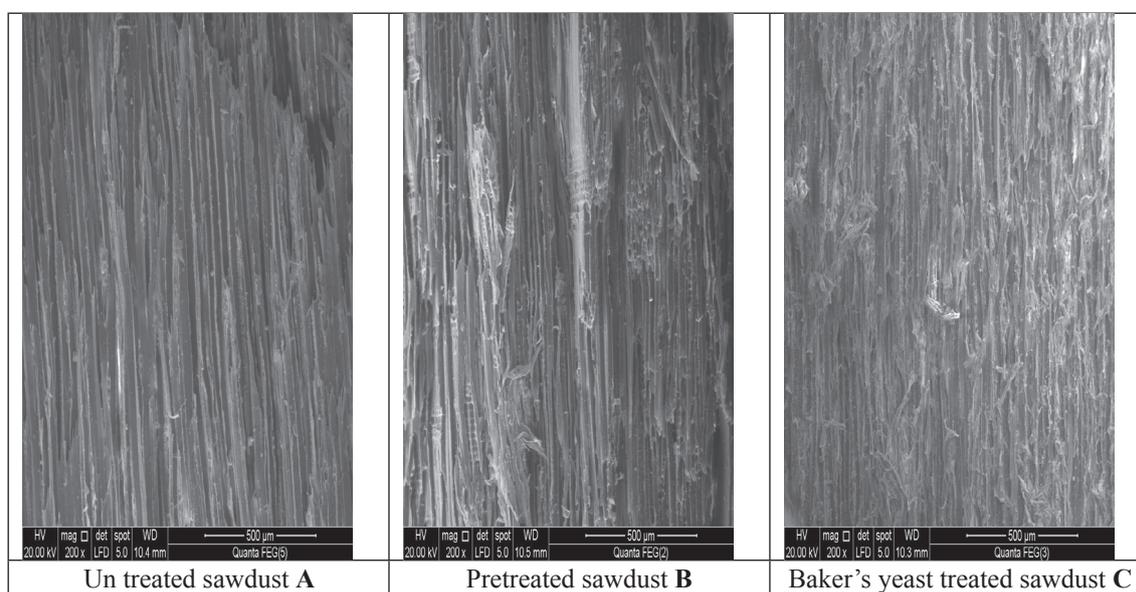


Fig. 4. Scanning electron micrograph for different samples of sawdust.

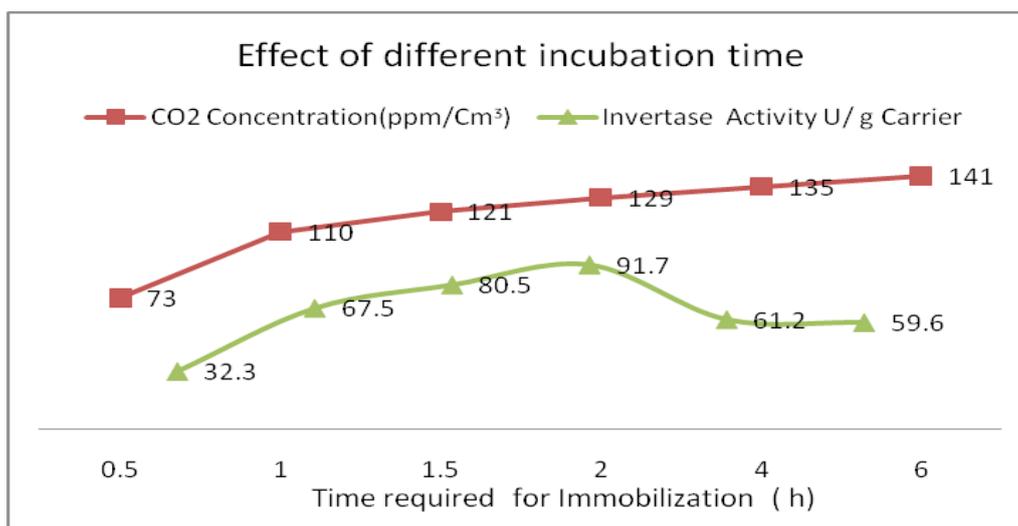


Fig. 5. Effect of different incubation time on invertase immobilization.

Effect of different type and concentrations of yeast

This experiment investigated how yeast type affects yeast performance in invertase immobilization. The effect of yeast type and its different concentrations on invertase immobilization was investigated by measuring invertase activity.

Baker's yeast is obtained in three common forms, compressed yeast, active dry yeast and instant dry yeast. Compressed fresh yeast examined and compared to the instant dried type while active dry yeast was excluded. And this was because it is covered by protective coating of yeast detritus in forms of granules dried less quickly than its instant dry counterpart in the form of small porous rods that can take up water more rapidly than granules [23]. Different concentrations of both type of yeast from 0.5 g up to 3.0 g/50 ml were used.

As shown from **Figure 6**, invertase was affected by the type of yeast and its concentration. Dry yeast succeeded to give higher invertase activity than compressed yeast. Cells of living yeast contain glutathione. Only become accessible once the yeast has died [24]. Because deactivated yeast had a high content of dead yeast cells, so it contained glutathione. The effect of the glutathione in invertase allowed for activation and stabilization [25]. This may explain the increment of invertase activity by increasing

the concentration of dry yeast. The increments of glutathione up to a certain limit (1.5g yeast) enhanced invertase above which the activity began to decrease. By using fresh compressed yeast, invertase activity increased that was might be due to more yeast resulted in more CO₂ production. From an economic point of view using dry yeast is more effective [26]. Concentration of 1.5g dry yeast gave nearly the same as invertase activity of 3.0 g compressed yeast (130 U/ g carrier). Dry yeast was preferable for enhancing invertase activity up to about 1.4 fold higher than compressed yeast at concentration (1.5 g).

Storage stability

The immobilized invertase was more resistance to dryness and had high storage stability as it retained 100 % of its activity after 4 months at 4 °C, while it retained 85% of its activity after storage of 3 years at the same temperature as shown in **Figure 7**. The immobilized invertase of this study showed superior storage stability compared to other immobilized invertase in the literatures. After 24 months of storage at 4 °C, 93% of the activity was retained, while invertase of other author [27] showed less storage stability and retained the same activity (93% of the initial activity) during 12 weeks of storage only. The immobilized invertase on date palm fibers retained 78 % of the activity for one year of storage at room temperature [28].

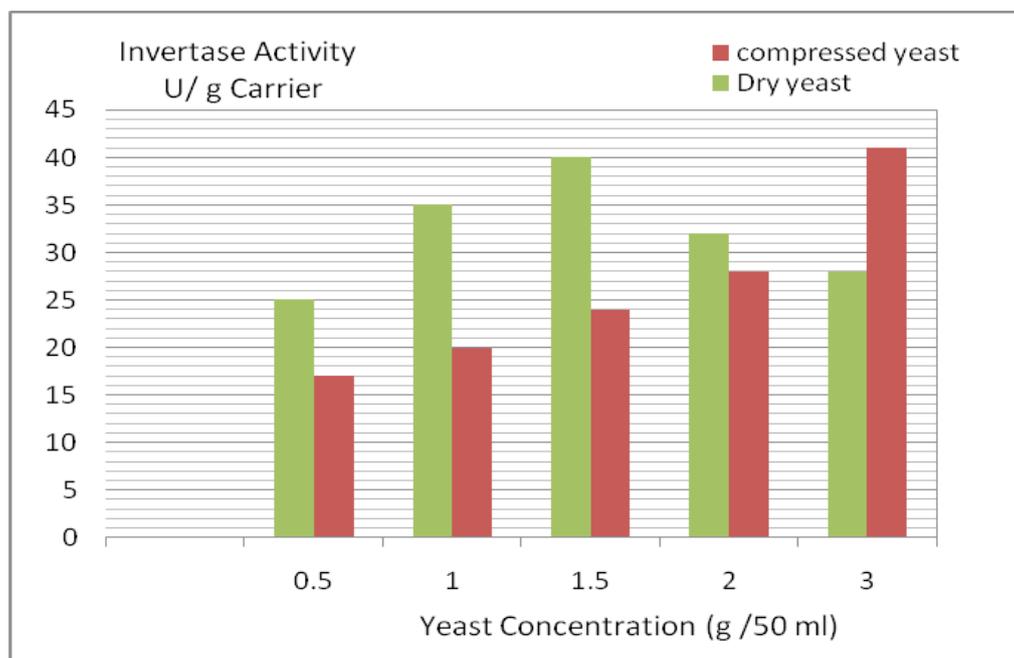


Fig. 6. Effect of different type and concentrations of yeast.

This high storage stability was most likely anticipated to that glutathione may stabilize invertase activity during storage and also revived enzyme activity which had become inactive with time [29]. However, whether the observed increase in storage stability was due to presence of glutathione in dry yeast or not it remains need proof and further experiments are needed. But it can be concluded that the technique of the present study provides vital insights on the role of immobilization technique on the stability and activity of the enzyme.

Sawdust characterization by (FT-IR)

Infrared spectroscopy (FT-IR) is important for analyzing the structural and chemical changes of wood components due to the different treatments to which wood is exposed [30-31]. The sawdust spectrum as in **Figure 8** showed just like all wood samples the same basic structure: wide

OH expansion (3300–4000 cm^{-1}), C–H extended in methylene and groups (2800–3000 cm^{-1}), and a strong wide overlap with sharp and separated absorptions in the wave length 1000 to 1750 cm^{-1} [32]. The fingerprint of cellulosic component is enclosed in a circle as shown in the figure. While those surrounded by the rectangle in the spectrum of treated sawdust represented the change due to binding of invertase. There were clear differences in infrared spectra, in the transmittance, absorbance, intensity and the bands shapes and in their position numbers for treated and pretreated sawdust. The stretched band at 1234.2189 cm^{-1} of pretreated sawdust spectrum represents O–H phenolic. This band had significantly narrowed in the spectrum of treated sawdust, which indicated removal of phenols [33]. This result confirmed the role of baker's yeast and CO_2 in the treatment technique.

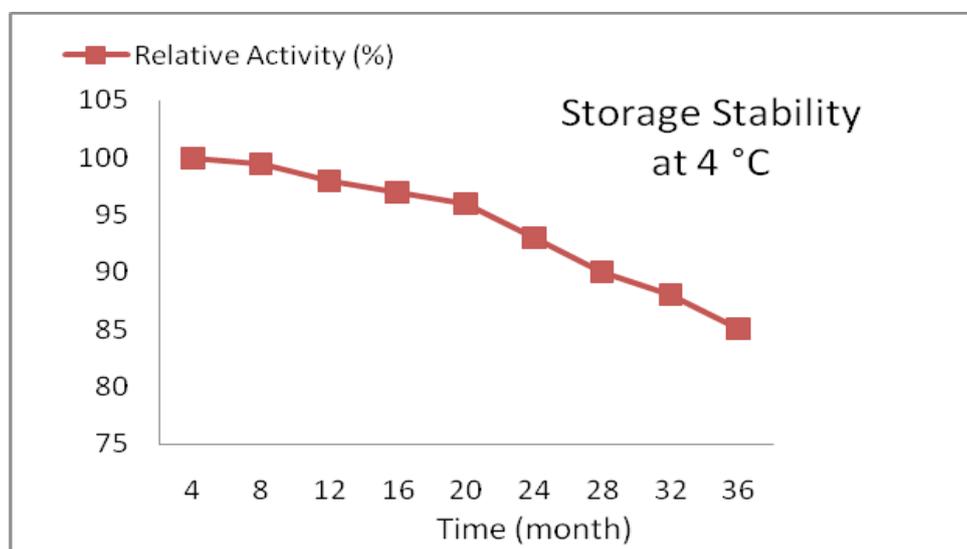


Fig. 7. Effect of storage stability on invertase immobilization.

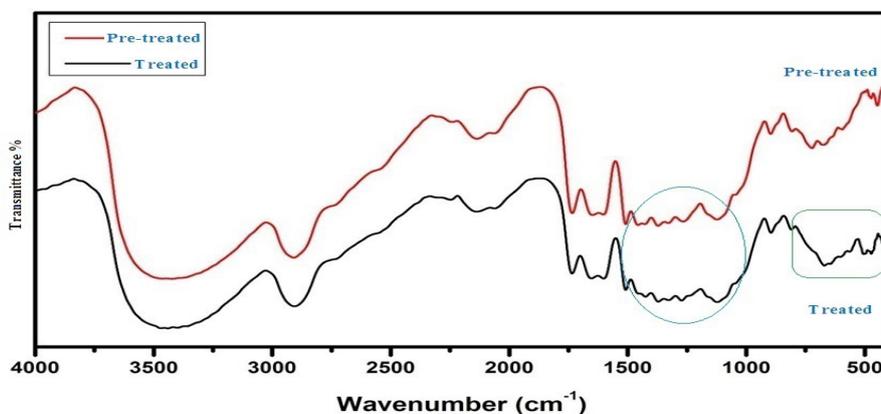


Fig. 8. (FT-IR) of pretreated and treated sawdust.

TABLE 2. Intensity of IR bands according to their functional groups.

Functional group	Intensity of treated sawdust with yeast	Intensity of pretreated sawdust	Band position (cm ⁻¹)
Holocellulose	63.3404	51.5067	1730.7991
CH ₂ cellulose	55.8953	47.098	1462.7422
CH ₂ cellulose	54.2102	47.1251	1425.1371
C–H cellulose, hemicellulose	53.5607	47.637	1384.6393
C–H cellulose, hemicellulose	53.3854	47.9473	1346.0699
O–H phenolic	Narrow band	-----	1260.2532
O–H phenolic	-----	Stretched band	1234.2189

Table 2 showed that the intensity of treated sawdust at all band positions exceeded the intensity of pre-treated sawdust. This also confirmed the changes caused by baker's yeast and CO₂. The absorption occurs in 1730 cm⁻¹, 1462 to 1425 cm⁻¹, 1384 to 1346 cm⁻¹ are caused by holocellulose, CH₂ cellulose, C–H cellulose and hemicellulose respectively. Each band position (cm⁻¹) represented specific functional group as shown in **Table 2** according to what reported by Jiangtao et al [34], who had the same results. From the increase in intensity, it can be concluded that the increase in the amount of various types of cellulose [35] enabled invertase to be immobilized on sawdust.

Enzymatic hydrolysis of sucrose

Sucrose hydrolysis was conducted by immobilized invertase at 50°C for different periods of time. The hydrolysis was monitored by percentage as follows :

Sucrose hydrolysis percentage = $\frac{\text{Total reducing sugar}}{\text{Initial sucrose concentration}} \times 100$

From **Table 3**, it was apparent that increasing reaction time up to 5 h influenced the sucrose hydrolysis. This result was similar to other works other than they used 60 °C for invertase activity [36 & 37]. By comparing the hydrolysis products of sucrose by immobilized invertase activity, it was observed that the products were the same over the first 4 hours. But after 5 hours, sucrose has almost disappeared and this indicated that sucrose completely converted to glucose & fructose. The products demonstrated by paper chromatography as mentioned before. On employing the descending chromatographic

technique and spraying with aniline hydrogen phthalate, Saccharides such as glucose, fructose and sucrose were observed **Figure 9**.

Enzymatic synthesis of oligosaccharides

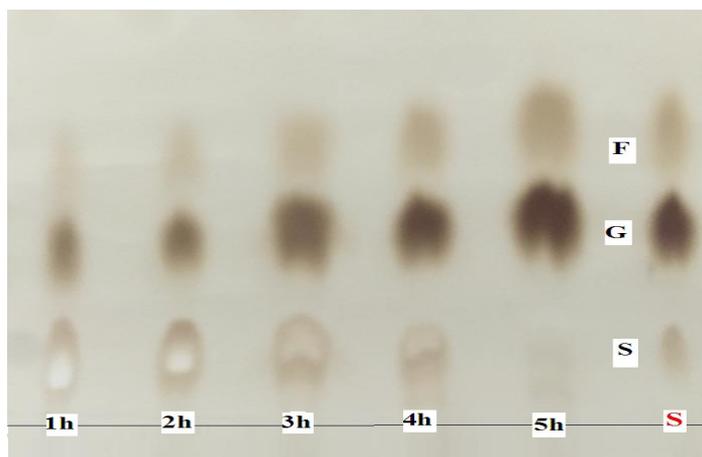
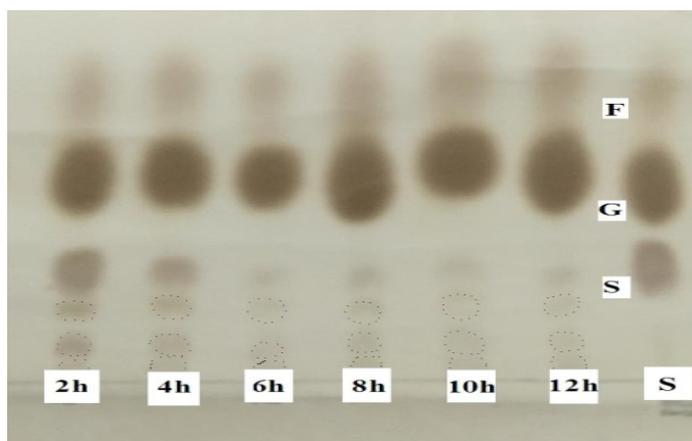
When repeating the same preceding experiment at a temperature of 20°C with a change in reaction times between 2 h to 12 h, two detectable spots & one faint spot were observed behind the sucrose spot. Those spots were indeed oligosaccharides **Figure 10**.

Old observations for the production oligosaccharides during the action of honey invertase and yeast invertase on sucrose were described [38]. Actually, the mechanism by which these oligosaccharides formed was unknown. Invertase catalyzes the hydrolysis of sucrose [39]. Oligosaccharides usually formed by transferase enzyme [40]. The author attributed this behavior to the fact that, it was documented that invertase can exhibit transferase activity under certain conditions. Some of these conditions were presence of surfactant and low water medium [41]. Surfactant formed reversed micelle by which invertase entrapped in and then exploited for fructo-oligosaccharides synthesis. In a similar way, sawdust offered protection for invertase and decreased the amount of water around it. This may explain why immobilized invertase on sawdust enhanced the accumulation of oligosaccharide especially at low temperature.

The three oligosaccharides formed by immobilized invertase at 20°C, have been confirmed by hydrolysis in 0.1N HCl, to be consists of glucose and fructose. Each hydrolysate examined chromatographically. This result was

TABLE 3. Effect of reaction time on enzymatic hydrolysis of sucrose.

Sucrose hydrolysis %	Invertase reaction time (h)
29	1
43	2
65	3
80	4
95	5

**Fig. 9. A chromatogram showing the hydrolysis products of sucrose by the action of immobilized invertase at 50°C****Fig. 10. A chromatogram showing the hydrolysis products of sucrose by the action of immobilized invertase at 20°C**

similar to that reported by [42] who stated that yeast invertase formed trisaccharid consists of two fructose and glucose molecules at 25°C. The optimum temperature for which the reaction rate of yeast invertase was at its highest is 60°C [43].

Limited articles are available about the synthesis of oligosaccharides by cold-adapted yeast invertase.

But in general, different techniques have been proposed for enhancing the expression of cold-

active enzymes [44]. The technique of the present study may improve functional adaptation for high invertase activity at low temperature. This may increase the opportunity for the discovery of new cold-adapted enzymes.

Conclusion

Based on the above study, the results clearly supported the possibility of using wood wastes for invertase immobilization. The changes of surface of wood wastes were due to effect of CO₂. The

new method is a low-cost and rapid technology to immobilize invertase and consequently produce oligosaccharides at low temperature. Sucrose hydrolysis percentage reached to 95 % after 5 hours of reaction at 50°C.

Recommendation:

The ability of wood wastes to absorb CO₂ from baker's yeast may open the way for new methods of CO₂ capture from atmosphere. Further study is required to outline how such an approach could actually be implemented. The use of wood waste for CO₂ capture may help in reduction of negative impact of CO₂ on global warming.

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تقنية صديقة للبيئة لتقييد الانفريز وتخليق الأوليجوساكاريد في درجات الحرارة المنخفضة

دعاء عبدالرحمن محمود

قسم كيمياء المنتجات الطبيعية و الميكروبية، المركز القومي للبحوث، الدقي – جيزة، جمهورية مصر العربية.

تم ابتكار تقنية صديقة للبيئة لتقييد الانفريز. إنها بسيطة للغاية ولا تحتاج إلى إعداد إنزيم نقي أو حامل غالي الثمن. تعتمد هذه التقنية على خلط نشارة الخشب المعالجة مسبقًا مع خميرة الخبز في نظام مغلق ومقارنتها مع نظيرتها في النظام المفتوح. تقدم هذه التقنية نظرة عملية جديدة حول دور ثاني أكسيد الكربون في التعديل المحتمل لسطح نشارة الخشب وتقييد الإنزيم. كما توفر رؤى حيوية حول الدور الرئيسي لتقنية التقييد على ثبات ونشاط الإنزيم. استعاد الانفريز المقييد نسبة ٩٣٪ من نشاطه بعد تخزينه لمدة عامين متتاليين عند ٤ درجات مئوية. على الرغم من أنه تم تقييد الانفريز على نسبة كبيرة من المواد السليلوزية المختلفة، إلا أن حداثة الدراسة الحالية تمثل التخليق الأنزيمي للأوليجوساكاريد بواسطة الانفريز على البارد عند درجة حرارة ٢٠ درجة مئوية.