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USE OF IMMOBILIZED L-ARABINOSE ISOMERASE FOR PRODUCTION OF TAGATOSE

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ABSTRACT

L-arabinose isomerase from *Escherichia coli* O157:H7 Was immobilized with activated Bentonite from local markets of Baghdad, Iraq by 10% 3-APTES and treated with 10% aqueous glutaraldehyde, the results refer that the yield of immobilization was 89%, and pH profile of free and immobilized L-arabinose isomerase was 7 and 7.5 and it is stable at 6-8 for 60 min respectively, while, the optimum temperature was 30 and 35°C and it was stable at 35 and 40°C for 60 min but it loses more than 60 and 30% from its original activity at 50°C for free and immobilized L-arabinose isomerase respectively. Immobilized enzyme retained its full activity for 32 day, but it retained 73.58% of its original activity after storage for 60 day at 4°C, and its retained a full activity for 36 continue usage; while it retained 84.63% of its original activity after 50 continue usage. Immobilized enzyme could to get about 85% of D-tagatose from 100 gm\L of D-galactose as a substrate with at 80 rpm of reaction speed for 24 hr.

Key words: immobilized enzyme, L-arabinose isomerase, bentonite, D-tagatose.

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استعمال أنزيم L-arabinose isomerase المقيد في إنتاج سكر التاكاتوز Tagatose

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الخلاصة

تم تقييد أنزيم L-arabinose isomerase المنتج من قبل بكتريا *Escherichia coli* O157:H7 بوساطة البنتونايت المستحصل عليه من الأسواق المحلية لمدينة بغداد والمنشط بوساطة محلول 10% 3-APTES والمعامل بمحلول 10% glutaraldehyde المائي، وبينت النتائج التي تم الحصول عليها أن حصيلة التقييد كانت 89% وان الاس الهيدروجيني الأمثل لفعالية الأنزيم الحر والمرتبط كانت 7 و7.5 وانه كان ثابتا في مدى تراوح بين 6-8 لمدة 60 دقيقة على التوالي، وبلغت درجة الحرارة المثلى للفعالية 30 و35م وانه كان ثابتا عند درجة حرارة 35 و40م لمدة 60 دقيقة، إلا انه فقد أكثر من 60 و30% من فعاليته الأصلية عند درجة حرارة 50م للأنزيم الحر والمرتبط على التوالي لمدة 60 دقيقة، واحتفظ الأنزيم المقيد بكامل فعاليته لمدة 32 يوما، إلا انه احتفظ بنحو 73.58% من نشاطه الأصلي بعد التخزين لمدة 60 يوما عند درجة حرارة 4م، كما واحتفظ بفعاليته بشكل كامل لـ 36 عملية استعمال مستمرة؛ بينما احتفظ 84.63% من فعاليته الأصلية بعد 50 مرة من الاستعمال، ولوحظ أن حصيلة إنتاج سكر التاكاتوز بلغت حوالي 85% من استعمال 100غم/لتر من سكر الكالاكتوز كمادة أساس بظروف تفاعل تشمل سرعة دوران مقدارها 80 دورة/دقيقة لمدة 24 ساعة.

الكلمات المفتاحية: الأنزيمات المقيدة، L-arabinose isomerase، سكر التاكاتوز، البنتونايت.

INTRODUCTION

D-tagatose is a rare natural hexoketose that has received generally recognized as safe (GRAS) certification from the U.S. Food and Drug Administration (FDA) and was allowed to be used in food and beverage industry, its sweetness is 92 % of sucrose when compared in 10 % solutions, which possesses a low caloric value (1.5 kcal g-1,38 % of sucrose), a low glycaemic index, prebiotic properties and shows non cariogenic properties, It has been considered as a promising sucrose substitute because it shows numerous advantages for human health such as low calorie, stabilizing blood sugar levels, preventing tooth decay, and promoting growth of the intestinal probiotics **(Van Holsbeeck *et al.*, 2014 ; Xu *et al.*, 2014)**. D-tagatose is found in oranges, apples, pineapples, sterilized cow milk etc. but not in sufficient amounts for commercialization **(Kim, 2004)**, Therefore, enzymatic method to industrial implementation by L-arabinose isomerase (AI) to production of D-tagatose **(Van Holsbeeck *et al.*, 2014)**. L-arabinose isomerase (EC 5.3.1.4), an aldo keto isomerase defined as a key enzyme in the microbial pentose phosphate pathway, which has been considered an important biological catalyst in food and pharmaceutical industries **(Xu, 2014)**, This enzyme could catalyzes the conversion of D-galactose to D-tagatose, as well as the conversion of L-arabinose to L-ribulose, Since it began commercial production of D-tagatose, many of the relevant information on L-arabinose isomerase has been recently documented in patents, that have been reported as D-tagatose isomerization enzymes, and a large number of these enzyme genes have been cloned from various bacterial species to use it for galactose isomerization to production of D-tagatose **(Bortone, 2013)**. Immobilized enzymes are used in many applications **(Al-Soufi, 2018)**, this technique was considered as one of the important methods that provides several advantages for enzymes **(Al-Soufi, 2016)**, such as, reducing cost production, promote and increase stability, improve catalytic properties and the possibility of using them more than once **(Inouye *et al.*, 2007)**, Several methods have been reports to immobilization of L-arabinose isomerase to production of D-tagatose, such as, covalent binding to agarose **(Kim *et al.*, 2001)**, alginate beads **(Oh *et al.*, 2001 ; Ryu *et al.*, 2003)**, Chitopearl beads **(Lim *et al.*, 2008)** and calcium alginate beads **(Qi *et al.*, 2015)**. Bentonite (Montmorillonite) clay is a 2:1 dioctahedral (smectites in general) that considered one of the most popular clay rocks in the world which widely used in many industrials applications, it use as a support materials in immobilized enzymes, given what it own of acidic nature which provide acid sites for binding of enzymes through NH_2 group **(Alsoufi, 2018)**, so, It is enough to adsorption of enzymes with clays, as well, clay can be activated and linked with glutaraldehyde to make covalently bond between clay and enzyme **(Al-Soufi, 2015)**. In this context, Bentonite clay has been used to immobilize many enzymes **(Al-Soufi, 2015; Alsoufi, 2018)**, so, this study aimed to use bentonite to immobilized L-arabinose isomerase, due to huge quantities of this clay in the Western Desert for Republic of Iraq and study some of its characteristics and its application for tagatose production.

MATERIALS AND METHODS

Source of Enzyme

L-arabinose isomerase (Chemily Glycoscience, USA) from *Escherichia coli* O157:H7 (1000 U/mg protein). One unit of enzyme activity was defined as the amount that production 1µg of D-tagatose\min at 30°C, pH 7 **(Kim *et al.*, 2001)**.

Source of Bentonite

Bentonite was obtained from local markets of Baghdad, Iraq.

Clay Activation

Bentonite was activated by stirring with 10% [(3-Aminopropyl) triethoxysilane] 3-APTES solution in acetone (v/v) for 1hr at room temperature, filtered out, washed with acetone and drying at 80°C. After that, treated with 10% aqueous glutaraldehyde solution (v/v) for 1hr, filtered out, washed and dried at room temperature, and stored in 20mM phosphate buffer pH 7.0 at 4°C until use **(Al-Soufi, 2015)**.

Enzyme assay

Free and immobilized enzyme activities were estimated by using D-galactose as substrate, the enzyme (2mL of free) or (1gm of immobilized) was incubated with 3mL of working solution (0.05M Tris-HCl buffer, pH 7 that containing 0.35gm\mL and 1mM of $MnCl_2.4H_2O$), the reaction was beginning by incubated of Mixtures for 2hr at 60°C in a water bath with shaking **(Van Holsbeeck *et al.*, 2014)**. Then, Tagatose concentration from free and immobilized enzyme was measured by a spectrophotometer at 560nm using the colorimetric cysteine-carbazole method **(Dische & Borenfreund, 1951)**.

Immobilization of L-arabinose isomerase:

10mL of enzyme solution (50U\mL) was mixed with 10gm of activated bentonite at room temperature for 2hr with slowly continuous stirrer **(He *et al.*, 2000)** then treated with 1% of glutaraldehyde in 50mM Tris/HCl buffer solution, pH 7, for 1hr **(Zhou & Xiao, 2001)**, the immobilized L-arabinose isomerase was stored in refrigerator until use.

Yield of immobilization

Immobilization yield was estimated by the difference between protein concentration (mg/mL) **(Bradford, 1976)** of enzyme solution that adds to activated bentonite (At_0) and same solution after stirring activated support with at 4°C for 24hr (*Att*). The immobilization yield (*IY*) was calculated according to following equation **Al-Soufi (2016)**.

$$IY\ (\%) = \frac{At_0 - Att}{At_0} \times 100$$

Characterization of immobilized L-arabinose isomerase

optimal pH of free and immobilized enzyme were estimated at 45°C by 50mM of maleate-NaOH, phosphate-NaOH, Tris-HCl and borate-NaOH buffer pH range (4.0-6.0), (6.0-7.0), (7.0-9.0) and (9.0-10.0), the effect of stability was estimated by pre incubated of enzyme for 1hr at 30°C in 50mM Tris-HCl buffer pH 8.0. The optimal temperature of free and immobilized thermolysin was determined at range from 25-50°C with 50mM Tris-HCl buffer pH 8, while, temperature effect on stability was estimated by pre incubated for 1hr at 25-50°C **(Yoon *et al.*, 2003)**. The effect of storage on enzyme and recycling according method of **Alsoufi (2018)**.

Tagatose production

The effect of galactose concentration (gm/L) as a substrate, rotation speed (rpm) and time (hr) on tagatose production were investigated in a bioreactor containing 300mL of 50, 100, 150, 200, and 250gm/L galactose, the rotation speed were 50, 60, 70, 80, 90 and 100rpm, while reaction times were 24, 48, 72, 96 and 120hr respectively **(Lim *et al.*, 2008)**. The reactions performed of D-Tagatose production in the bioreactor at 35°C, 50mM potassium phosphate buffer pH 7.5, 80rpm and 100gm/L of immobilized L-arabinose isomerase.

RESULTS AND DISCUSSION

Yield of immobilization

The yield of immobilized L-Arabinose Isomerase by with bentonite was 89%, which considerer encouraging for dependence it in this study, in this field many researchers use

different matrix to immobilized L-Arabinose Isomerase, such as, calcium alginate beads treated with glutaraldehyde **(Oh *et al.*, 2001)**, covalent binding to agarose **(Kim *et al.*, 2001)**, alginate beads **(Kim *et al.*, 2003)**, chitopearl beads **(Lim *et al.*, 2008)**, and copper-chelate Eupergit C250L **(Bortone, 2013)** which get of bind rate 33.9mg of L-arabinose isomerase from *Thermotoga maritima* per one g of dry support as immobilization yield.

This step in enzyme immobilization is considered a very important to ensure immobilized a large amount of enzyme to bentonite which use to production tagatose from galactose by immobilized L-arabinose isomerase.

pH

The results for pH profile of free and immobilized L-arabinose isomerase refer that it 7 and 7.5 (Figure 1) and it is stable at 6-8 for 60min (Figure 2) respectively.

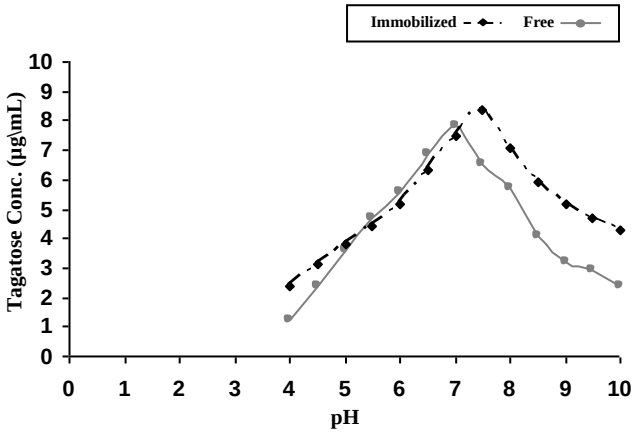

Figure (1): Optimum pH of activity for free and immobilized L-arabinose isomerase.

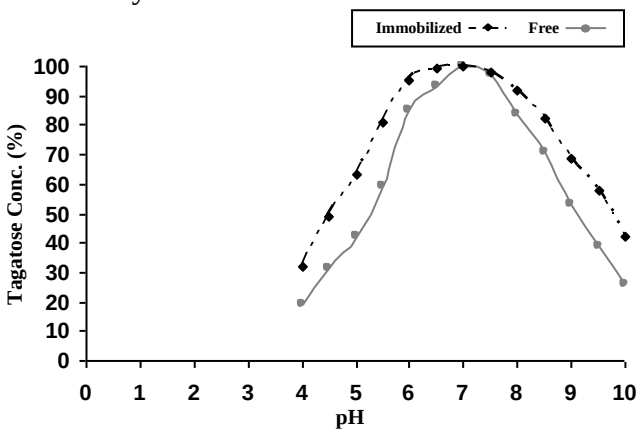

Figure (2): Optimum pH of stability for free and immobilized L-arabinose isomerase.

Many authors have reported of this effect, such as **Kim *et al.* (2003)** that found the optimum pH for free and immobilize L-arabinose isomerase with alginate beads was 7.5 and 8.0 respectively, while, **Lim *et al.* (2008)** and **Bortone (2013)** observe that pH activity was 7.5 for immobilize L-arabinose isomerase by chitopearl beads and copper-chelate Eupergit C250L, in this context, **Qi *et al.* (2015)** explain that optimum pH for most of prokaryotes were between 6.0-8.0. Also, **Nguyen *et al.* (2018)** refer that the optimal temperature of L-arabinose isomerase from *Clostridium hylemonae* was 7.5 and enzyme was most stable at pH 6.5-7.

The deviation of optimum pH profile for immobilize enzyme may lead to negative effect on enzyme activity, so, the feasibility of immobilization will end, and using of free enzyme will it be benefit economic **(Al-Soufi, 2015; Al-Soufi, 2016)**.

Temp

The optimum temperature was 30 and 35°C (Figure 3) and it was stable at 35 and 40°C for 60min but it loses more than 60 and 30% from its original activity at 50°C (Figure 4) of free and immobilized L-arabinose isomerase respectively.

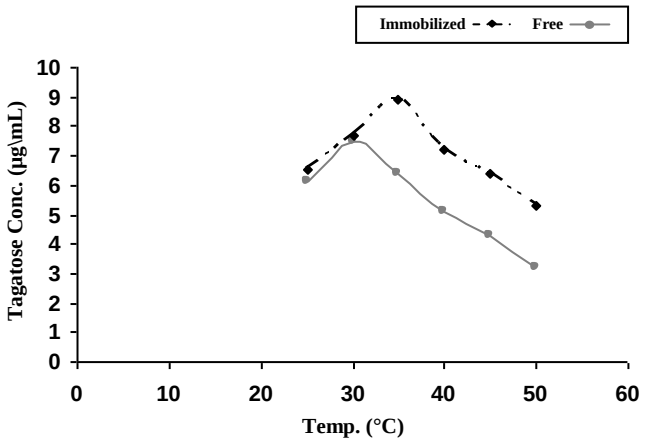

Figure (3): Optimum temperature of activity for free and immobilized L-arabinose isomerase.

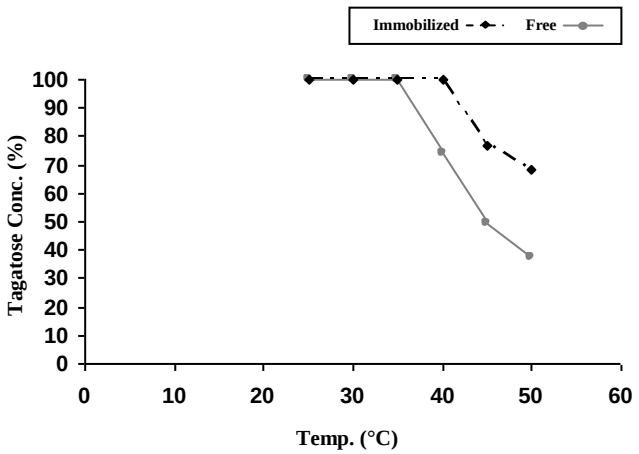

Figure (4): Optimum temperature of stability for free and immobilized L-arabinose isomerase.

In this subject, **Kim *et al.* (2003)** refer that the temperature activity was 60 and 65°C for free and immobilized L-arabinose isomerase with alginate beads respectively, while, **Lim *et al.* (2008)** observe that maximum activity of immobilized enzyme by chitopearl beads was between 90 and 95°C. Also, **Bortone, (2013)** found that The optimum temperature of immobilize L-arabinose isomerase by copper-chelate Eupergit C250L was 80°C, On this basis, **Qi *et al.* (2015)** explain the differences in optimum temperature of enzymes are due to enzyme

sources, it was be 30-50°C for mesophiles, 60-80°C for thermophiles and 85-90°C for hyperthermophiles. On the other side, the optimal temperature of L-arabinose isomerase of *Clostridium hylemonae* was 50°C and enzyme showed high remarkable stability with 90 and 85% of its initial activity at 45°C and 60°C respectively **(Nguyen *et al.*, 2018)**.

All of studies are referring to improve of thermal activity and stability for enzymes for limited temperature, then increasing temperature will decreasing enzyme activity due to denature the enzymes through its effect on open enzyme folds thus exposure it content from amino acids to the reaction medium **(Alsoufi, 2016)**.

Storage and Reuse

immobilized L-arabinose isomerase retained its full activity for 32 day, but it retained 73.58% of its original activity after storage for 60 day at 4°C (Figure 5), and its retained a full activity for 36 continue usage; while it retained 84.63% of its original activity after 50 continue usage (Figure 6).

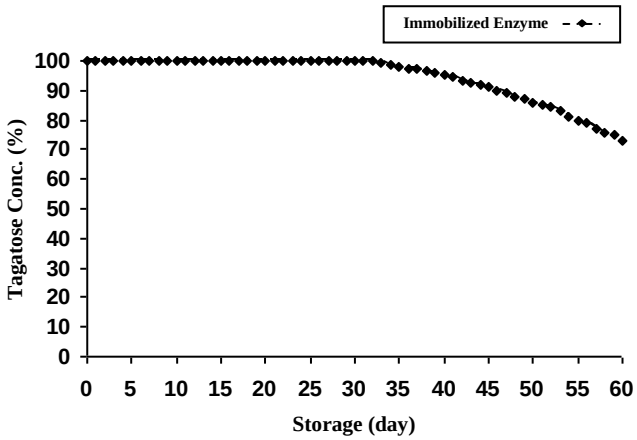

Figure (5): Storage stability of immobilized L-arabinose isomerase.

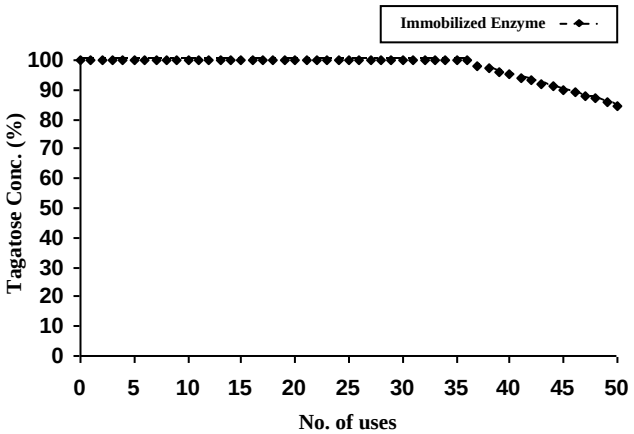

Figure (6): Reuse effect of activity for immobilized L-arabinose isomerase.

In this regard, **Kim *et al.* (2001)** refer that immobilized L-arabinose isomerase was stably produced an average of 7.5gm tagatose/L. day for 7 day, while, **Oh *et al.* (2001)**, found that the immobilized L-Arabinose isomerase with a packed bed reactor produced an average of 30gm tagatose/L.day from 100gm galactose/L for 8 day. Also, **Ryu *et al.* (2003)**, observe that

the half-life of tagatose production by immobilized thermostable L-arabinose isomerase with alginate was 24 day, while, **Bortone (2013)** noted that the time course of D-tagatose production by immobilized L-arabinose isomerase with copper-chelate Eupergit C250L did not lose it's activity after 242hr at temp. 60°C.

The storage stability and reuse of immobilized enzymes represents one of the main economic factors when thinking about immobilization because it gives a clear conception about the efficiency of materials which are used for this purpose **(Alsoufi, 2016)**.

Application

It was used immobilized L-arabinose isomerase on bentonite use for production of D-tagatose from D-galactose in this study, the results refer that yield of D-tagatose was 42.6, 85.3, 85.7, 86.1 and 86.4 % at 50, 100, 150, 200 and 250 D-galactose concentration respectively (Figure 7). The effect of time on yield production refer that D-tagatose was 52.7, 84.2, 84.4, 84.5 and 84.6% at 24, 48, 72, 96 and 120hr of reaction time respectively (Figure 8). While the effect of reaction speed referred that D-tagatose were 30.8, 40.8, 63.3, 84.7, 84.8 and 84.9% at 50, 60, 70, 80, 90 and 100rpm respectively (Figure 9). So, the optimum result refer that use of 100gm\L of D-galactose as a substrate with immobilized L-arabinose isomerase on bentonite by shaking 80rpm for 24hr lead to get about 85% of D-tagatose.

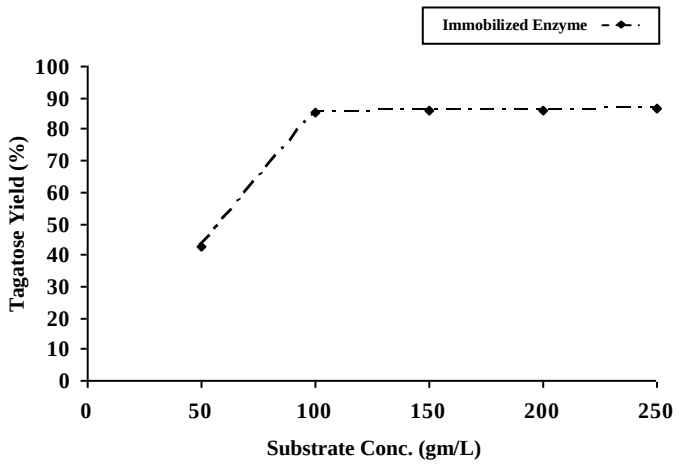

Figure (7): Effect of substrate concentration (gm/L) for tagatose yield (%) by Immobilized L-arabinose isomerase on bentonite.

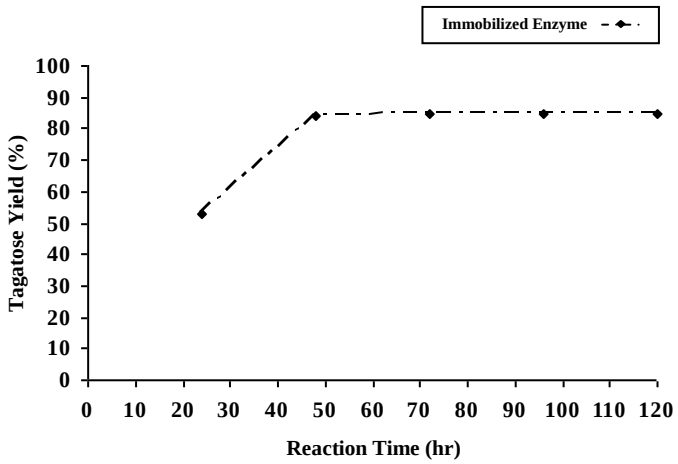

Figure (8): Effect of Reaction Time (hr) for tagatose yield (%) by Immobilized L-arabinose isomerase on bentonite.

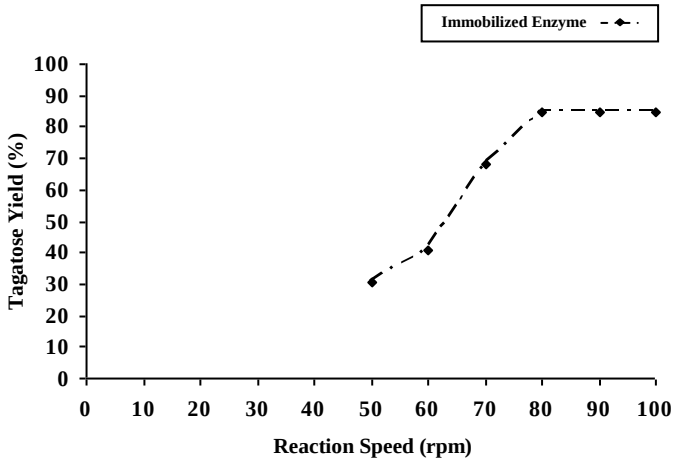

Figure (9): Effect of Reaction Speed (rpm) for tagatose yield (%) by Immobilized L-arabinose isomerase on bentonite.

Most researchers in this field (L-arabinose isomerase applications) are reported for this effect, such as **Kim *et al.* (2001)** that use a scaled-up immobilized enzyme system for immobilize L-arabinose isomerase of *Escherichia coli* by using covalent binding to agarose for produce D-tagatose, and they get an 99.9gm tagatose/L that produced from galactose with 20% equilibrium in 48 hr, While, **Kim *et al.* (2002)** use of free L-arabinose isomerase from *Thermotoga neapolitana* for production of D-tagatose from D-galactose and he observed that high conversion ratios was 68% at reaction temperature of 80°C, while, a lower conversion was observed at 90°C, due to loss of enzyme activity during the reaction, also, **Ryu *et al.* (2003)** founded that it can be produce D-Tagatose by using immobilized thermostable L-arabinose isomerase in alginate with 300gm/L D-galactose solution as a substrate to get about 145gm/L tagatose with an average productivity of 54 gm tagatose/L and an average conversion yield of 48% (w/w). Moreover **Lim *et al.* (2008)** produced 138gm/L of tagatose by using immobilized L-arabinose isomerase with chitopearl beads with pH control at 7.5 and 70°C in a stirred tank reactor containing 300gm/L galactose as a substrate. Also, **Bortone (2013)** use of immobilized L-arabinose isomerase by copper-chelate eupergit C250L for production of D-tagatose and refer that D-galactose conversion and average productivity were 44.4% and 0.02gm/lhr or 29.1% and 0.06gm/lhr at 4.5gm/L or 18gm/L initial D-galactose concentration, respectively. Whilst, **Nguyen *et al.* (2018)** referred that the bioconversion yield of D-tagatose by use free L-arabinose isomerase from *C. hylemonae* at 60°C reached approximately 46% from 10 mM of D-galactose after 2hr.

The balance point of temperature and pH in industrial application is very important to get a good product, the increase of temperature for reaction of enzyme for D-tagatose production, lead to increase of D-galactose conversion to D-tagatose due to increase the reaction rate and binding capacity of L-AI of the substrate until it reached the maximum reaction, so, high temperature more than 80°C will cause browning reaction and also high pH more than 9.0 lead to nonspecific reactions and browning reaction and correspond to the pH of the hydrolysis of lactose that is used as raw material **(Qi *et al.*, 2015)**.

Conclusions

This study show that possibility to immobilized L-arabinose isomerase with bentonite by glutaraldehyde and able to use the immobilized enzyme for many times without any loss of its activity.

Recommendations

Use immobilized L-arabinose isomerase for production of D-tagatose from D-galactose with highly efficient by using bentonite as a support material.

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