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Chemical Characterization of Levan and Optimization of immobilized *Bacillus tequilensis* levansucrase onto κ-Carrageenan –CMC Gel Beads

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BACILLUS tequilens was a good levansucrase producer (222.2 U/mL) with levan yield (130 g/L). The levan yield was characterized by FT-IR and the results recorded that the product was mainly fructose. Levansucrase produced by *Bacillus tequilens* was immobilized by covalent binding on κ-carrageenan and carboxy methyl cellulose gel beads activated by two-step method; the gel beads were soaked in polyethylenimine followed by glutaraldehyde. Then 2^2 full-factorial central composite experiment design was employed to optimize the conditions for the maximum enzyme loading efficiency to reach (14.01852 U/g gel beads). The free enzyme showed optimum activity at pH7 while, immobilization process increased the tolerance of enzyme at both acidic range pH3 and alkaline range pH10. The apparent K_m after immobilization was 2،85 mg/mL compared to 2.5 mg/mL for free enzyme. Maximum velocity V_{max} was 71.4 mg/min for free enzyme while it was 62.4 mg/min for immobilized formula of enzyme. An inhibition of enzyme activity was recognized with all tested metal ion as well as EDTA for either free or immobilized formula of levansucrase.

Keywords: Levansucrase, Immobilization, Response surface methodology, *Bacillus tequilens*.

Introduction

Levan is a kind of natural homo-polysaccharides. Its main structure consisted of sucrose molecule. It is elongated by a chain of fructosyl units connected through β-(2 → 6) linkages [1]. Levan industrial applications have been known as an emulsifier, formulation aid, stabilizer and thickener, also, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances were proposed [2]. Furthermore, levan is used in medicine field as plasma substitute, antihyperlipidemic, agent drug activity prolongator and antihyperlipidemic agent [3].

Levansucrases (LSs) (E.C.2.4.1.10), identified as a fructosyltransferases subclass, few years ago, it gained more interest, due to its ability to use the free energy to cleavage the non-activated sucrose for transferring the fructosyl group to a variety of acceptors such as mono- (exchange), oligosaccharides (FOS synthesis) or the growing fructan chain called (polymer synthesis) [4]. Accordingly, β (2→6) levan type FOSs, yielded through LS-catalyzed transfructosylation, characterized by important prebiotic effects that surpass current commercial (2→1) inulin type FOSs [5]. Furthermore it showed excellent water-holding capacity and protecting effect against anticancer [6], β-(2→6) levan polymers have shown antitumor and antidiabetic activities [7,8]. Hence, microbial LSs are of high interest as biocatalysts for the catalytic synthesis of novel type FOS prebiotics. As well as levan for food, cosmetics and pharmaceutical fields [9,10].

The application of immobilized enzymes in industrial processes is more demanded than the free forms as they help in the separation of reactants and products. Also, it allows the enzyme recovery for different cycle; furthermore it increased the enzyme stability and reduced the cost process. Additionally, immobilization increases the enzymes selectivity and reduces the inhibition caused by the products [11,12].

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Varieties of immobilization techniques were previously implemented for enzyme immobilization, like gel entrapment [13], cross-linking [14,15], physical adsorption [16] and covalent binding. Many authors have worked for a lot of years on enzyme covalent binding immobilization using natural hydrogels and performed different modifications on gel formation to increase the enzyme resistance to high temperature and also for improving the enzyme reusability and shelf stability [17,18].

The response surface method (RSM) is a mathematical and statistical combination technique for experimental designing. It is model planning to study the effects of some factors to reach the optimum conditions for enzyme production. The RSM was identified as a proper method detecting the optimal conditions or the region that fits the operation specification. The optimum conditions for enzyme immobilization have been reported previously [19].

In the present work, *Bacillus tequilensis* levansucrase was recorded as levansucrase producers. The yielded levan was characterized by FT-IR and paper chromatography. It was immobilized on modified grafted carrrageenan -CMC gel beads. $A2^2$ factorial design was done for the planned statistical optimization of immobilization. Comparative studies between the properties of both free and immobilized enzyme were studied.

Materials and Methods

All experiments were Carried out in triplicate and data are means ±SD (n = 3).

Chemicals

(κ-Carrrageenan) and *Carrboxy methyl cellulose* (CMC) were purchased from Sigma, Polyethylenimine (PEI) (MW: 423), Cat # 468533, was obtained from Aldrich and, sucrose was obtained from Sigma. Crude levansucrase was prepared in our laboratory. Other chemicals were of Analar or equivalent quality. Innotech Encapsulator, model IE-50, was purchased from Innotech Encapsulator in Switzerland.

Microorganism and its maintenance media

Bacillus tequilensis used in this study as a source for levansucrase. *B. tequilensis* was isolated from commercial pastry date samples collected from Elmadeena Elmonowra, Saudi Arabian. The bacterial isolate was routinely grown on nutrient (NA) agar medium at 37 °C and preserved at – 80 ºC in 50% (v/v) glycerol.

Cultivation conditions and crude enzyme extraction

Inoculation Medium and Fermentation.

An equal volumes of 50 mL of medium consisted of (g/L) (Sucrose, 80; yeast extract, 1; K_2HPO_4, 1 $MgSO_4$, 0.2 and pH 7) were autoclaved (20 min, 121 °C) and inoculated with 2 mL of 24h old inoculum prepared by inoculating a well growth slant of *B. tequilensis* into Erlenmeyer flasks containing 50 ml of nutrient broth medium. Flasks were incubated at 37 °C for 24h on a rotary shaker (200 rpm). At the end of incubation period, culture was centrifuged at 5,000 rpm for 15 min, 4°C and the culture filtered was used as a source of levansucrase. Partial purification of *Bacillus tequilens* levansucrase was done using different ethanol concentrations. Ethanol fraction at 30% was used for further during this study.

Levan identification.

FT-IR spectroscopy.

FT-IR (Bruker Vectra 22) Spectrometer equipped with a Dura Sample IR II™ detector used for characterization and identification of extracted levan powder with a spectral resolution of 4 Cm-1 with 400- 4000 Cm^{-1}.

Total carbohydrate quantative and qualitative determination

Determination of total carbohydrates by acid hydrolysis.

Complete acid hydrolysis of the sample polysaccharides were carried out according to the modified method by [20]. Total carbohydrate was determined in the extract using phenol-sulfuric acid method [21]. After suitable dilution, 1 ml 5% phenol solution was added to 1 ml of the resulted diluted solution. After mixing, 5 ml conc. H_2SO_4 was added rapidly to the mixture, shacked and set aside for 10 min at room temperature, then at 20-30 °C (in a water bath) for 20 min. Thereafter, the color density was measured at 490 nm Spectrophotometer UNICO 7200.

Qualitative examination of the hydrolyzed levan.

This was performed by chromatography of the resulted hydrolyzate on Whatman No.1 filter paper, using the solvent system: n-butanol-acetone-water (4:5:1) [22]. Authentic samples of D-glucose and D-fructose were co-chromatographed as reference substances. After chromatographic separation, the chromatogram was air dried and dipped in 40-50 ml of the color reagent (1.66 g of O-phthalic acid and 0.91 ml aniline were dissolved in a mixture

of 48 ml n-butanol, 48 ml diethyl ether and 4 ml water), air dried, and then heated at 105 °C for 10 min in an oven for developing the colored spots.

Quantitative Determination of the Hydrolyzed levan.

Quantitative determination of the hydrolyzed sugars was done according to the modified method of [23]. The individual chromatographic spots were cut off, divided into small strips, and dropped into 4 ml eluting agents (This consisted of 0.7 N HCL in 80% ethanol (v/v). It was prepared by adding 29 ml 36% HCl to 420 ml 95% ethanol and the mixture was made up to 500 ml distilled water) in test tubes and shaked for complete elution. The absorbances of the resulting colored solutions were determined at 390 nm. The quantities of sugars were determined by comparison to appropriate standard curves constructed under the same conditions. [24].

Degree of polymerization (DP)

DP was determined according to the following equation:

MW of native levan / MW of monomers (glucose + fructose).

Determination of molecular weight

Different concentrations of levan were prepared, and the flow time of equal volumes for each concentration at 30 °C was determined in a U-shaped Ostwald viscometer. Flow time of the same volume of distilled water was also determined as control. Thus, specific viscosity/C (gsp) was estimated.

Levansucrase immobilization

Preparation of κ-carrageenan and carrboxy methyl cellulose (carr. CMC) beads

For gel beads formation solutions of κ-Carrageenan (Carr) and Carrboxy methyl cellulose (CMC) in a concentration of 2:1 % (w/v) respectively were mixed together. The Carr-CMC solution was dropped through a nozzle of 300 μm using the Innotech Encapsulator in a hardening solution containing 2 % (w/v) $CaCl_2$ (Ca^{2+}) and was soaked for 3 hrs. The generated gel beads will then soaked in 4% PEI for 3 hours, then washed with distilled water and followed by soaking in 2.5% glutaraldehyed for 3 hours and finally washed with distilled water. The generated gel beads become ready for enzyme immobilization process.

Optimization of the enzyme loading capacity and loading time using grafted Carr -CMC gel beads using 2^2Full-Factorial central composite experimental design.

Optimization of loading capacity and loading time of partially purified levansucrase on the Carr. CMC gel beads were carried out by using 2^2 full-factorial central composite design [19,25] with four-star points ($\pm\infty$) and three replicates at the center point. Design matrix of 11 trials experiment (Table 2) shows the coded and actual values. The independent variables, loading time (X_t) and enzyme units' solution (X_U) were fitted with second order polynomial function to correlate the relationship between independent variables and response of immobilized units per gram gel disks/ beads as follow:

$$Y_{Activity} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_1 \beta_2 X_1 X_2$$

where, $Y_{Activity}$ is the predicted amount of levansucrase per gram gel beads, i.e., U/g beads. β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and $\beta_{22}$are quadratic coefficients, β_{1}, is cross product coefficients.

Statistical software SPSS (version 16.0) was used for the regression analysis of the experimental data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficients to determine each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6). Experiments were performed in triplicate and mean values were given.

Comparative studies between free and immobilized levansucrase.

Effect of pH on immobilized and free levansucrase

To determine the optimum pH for the free and immobilized levansucrase, the enzymes were incubated at 37 °C for 30 min into 2 mL of 1% (w/v) sucrose dissolved in different buffers in 0.2 M to cover the pH ranges (from 3.0 to 10.0), The data were normalized to 100% activity. The highest enzyme activity is expressed as 100%, and each pH is expressed relatively as a percentage of the 100% activity.

K_m and V_{max} of free and immobilized levansucrase

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of free and immobilized enzyme were calculated using Line weaver–Burk Plot. Sucrose was employed as a substrate (5–50mg/ml) dissolved in sodium acetate buffer (0.2 M, pH 5.0) at constant pH and temperature.

The Line Weaver-Burk plot (double reciprocal) method was used to obtain the Michaelis-Menten kinetic models adequate for the description of the hydrolysis of sucrose by the free and the immobilized enzyme. Apparent K_m and V_{max} of free and immobilized levansucrase were determined by plotting 1/[S] against 1/ [V], respectively.

$[S]/V_o = 1/V_{max} *[S] + K_m/V_{max}$ Where, [S] is the substrate concentration (sucrose), V_o is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant and is defined only in experimental terms and equals the value of [S] at which V_o equals $1/2V_{max}$.

Effect of metal ions on free and immobilized levansucrase.

To study the effect of metal ions and EDTA on free and immobilized levansucrase the different metal ions were added to the reaction mixture [2 mL of 1% (w/v) sucrose dissolved in different buffers in 0.2 M sodium acetate buffer pH 5.0] to reach to a final concentration of 0.02 Molar. Enzyme was incubated at 37 °C for 30 min. Enzyme activity without metal ion addition is expressed as 100 % and enzyme activity with addition of metal ion is expressed relatively as a percentage of the 100 % activity.

Results and Discussion

Bacillus tequilens was characterized as good levansucrase producers (222.2 U/mL). The yielded levan (130 g /L) was identified by paper chromatography and FT-IR. The results referred that the levan backbone was mainly fructose with traces of glucose (Fig. 1).

The FT-IR spectroscopy analysis of levan

FT-IR analysis was performed to confirm the levan produced by *Bacillus tequilens* as shown in Fig1. The IR spectrum showed high similarity in the pattern of peak absorbance. The levan has broad absorption at the wavenumber range of 3200–3600 cm^{-1} corresponding to the stretching vibration of the –OH group. Levan also exhibited absorption at the wavenumber range of 2800 - 3000cm^{-1} corresponding to stretching vibrations of C–H. The fingerprint of β- glycoside bond was found at 2090 cm^{-1}. The characteristic band at 1640 cm^{-1}, caused by vibration of C=O. The region of typical carbohydrate at the finger prints wavenumber range of 1000-1200 cm^{-1}. These results were agreement with Daris Qodarisman Nasir et al. [26].

Physiochemical analysis of levan.

Total carbohydrate of levan product was 97% quantity. Levan monosaccharide qualitative analysis was determined to be 6% glucose and 94% fructose. Molecular weight also was determined 64 KDa. The degree of polymerization (DP) was calculated using molecular weight to be 36.

Optimization of the enzyme loading capacity and loading time using grafted Carr –CMC gel beads using 2^2Full-Factorial central composite experimental design.

The results in Table 1 represented the design matrix of 11 trial experiments of 2^2 full-factorial central composite design. The results showed that maximum enzyme activity 14.01852 U/g beads were recorded in trial No.4 when 24 U of levansucrase incubated with one gram of Carr-CMC gel beads for 20h. This result was very similar to the predicted value obtained by the polynomial equation (13.68498 U/g beads).

The results obtained by 2^2 full-factorial central composite design were analyzed by standard variance analysis (ANOVA) as shown in Table 1. The second-order regression equation provided the levels of enzyme activity as a loading time function (X_t) and loading amount of units (X_u) which could be predicted by the following equations:

$$Y_{Activity} = 1.671417 + 0.051570X_1 + 0.278067X_2 + 0.004207X_{12} - 0.018277X_{22} + 0.021046X_1X_2$$

Table 2 showing the ANOVA results of levansucrase immobilized onto gel beads where the amount of enzymes loaded onto the gel beads ($Y_{Activity}$) were evaluated according to the ANOVA results and the polynomial equation. The F value was 16.82779 which referred to the model significant. Model terms have values of Prob > F (0.003) less than 0.05. This result which was considered significant. ANOVA indicated that the R^2 value of R^2 0.943, for response $Y_{Activity.}$ It was indicated that 94.3% variability data could be interpreted by the model. The observed R^2 was in reasonable agreement with the adjusted R^2 of 0.887. This result confirmed a satisfactory adjustment of the quadratic model of the experimental data [19]. All the previous results reflected the applicability and accuracy of the central composite design for optimization of levansucrase immobilization process. The data in Fig. 2 indicated a firm relation between the predicted and the observed results. Three-dimensional response surfaces (Fig. 3) were plotted on the model equation basis.

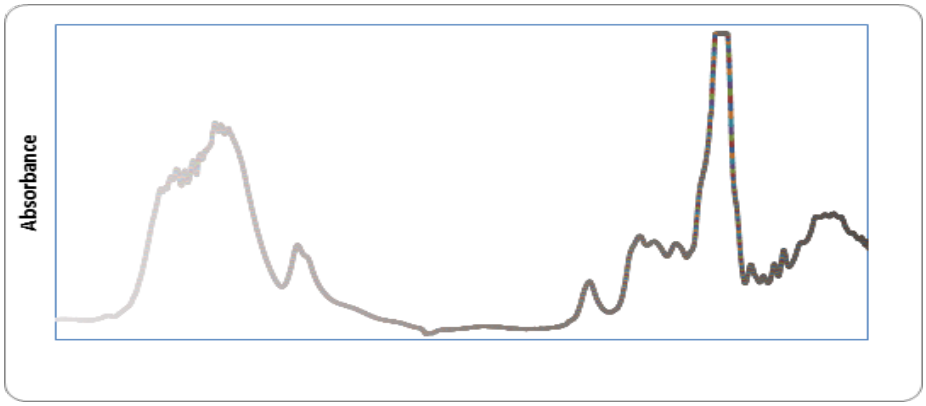

Fig. 1. FT-IR absorption spectra of levan.

TABLE 1. Optimization of the enzyme loading capacity and loading time using grafted Carr -CMC gel beads using 2^2 Full-Factorial central composite experimental designs.

Trial number	X_t	X_u	Levansucrase activity (U/g beads)	
	Time (h)	Units (%)	Observed values	Predicted Values
1	-1(8)	-1(12)	4.96296	5.97129
2	-1(8)	1(24)	9.01852	10.42786
3	1(20)	-1(12)	7.33333	6.19775
4	1(20)	1(24)	14.01852	13.68498
5	- ∞(4)	0(18)	7.68519	6.29782
6	+∞(24)	0(18)	8.12963	9.20081
7	0(16)	- ∞(6)	3.74074	3.92294
8	0(16)	+ ∞(30)	17.29630	16.87696
9	0(16)	0(18)	9.40741	9.79418
10	0(16)	0(18)	10.09259	9.79418
11	0(16)	0(18)	10.27778	9.79418

Actual values are between parentheses

TABLE 2. Model coefficients estimated by multiples linear regression (significance of regression coefficients).

Variables	Regression coefficients	Standard error	t- test	P-value	
Intercept	1.671417	6.097929	0.27410	0.794974	
x_t	0.051570	0.396979	0.12991	0.901705	
x_u	0.278067	0.459384	0.60530	0.571397	
x_{tt}	0.004207	0.008095	0.51967	0.625471	
x_{uu}	-0.018277	0.012223	-1.49526	0.195088	
x_{tu}	0.021046	0.017118	1.22950	0.273580	
ANOVAs					
	df	*SS*	*SM*	*F test*	*Significance F (P)*
Regression	5	137.2728	27.45456	16.82779	0.003
Residual	5	8.1575	1.63150		
Total	10	145.4303			

x_t is thecodded *value of loading time;*x_u codded value of loadingunits;*df* Degree of freedom; *SS* Sum of squares; *MS* Sum of squares; *F*Fishers's function; *Significance F(P)* corresponding level of significance. R^2 0.943, Adjusted R^2 0.887

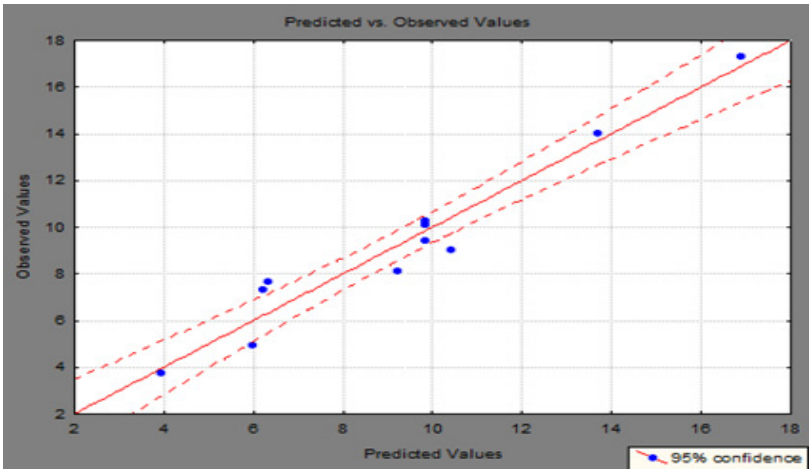

Fig. 2. A relation between observed and predicted results obtained by (CCD) at 95% confidence for optimization of loading time and loading units of levansucrase immobilization.

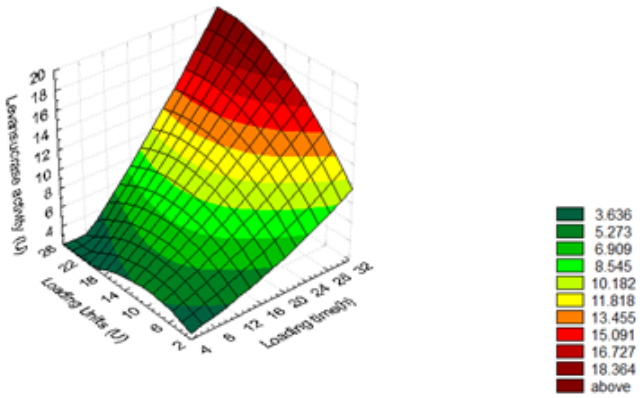

Fig. 3. Response surface plot showed the effect loading time(X1) and loading units (X2) time on levansucrase activity.

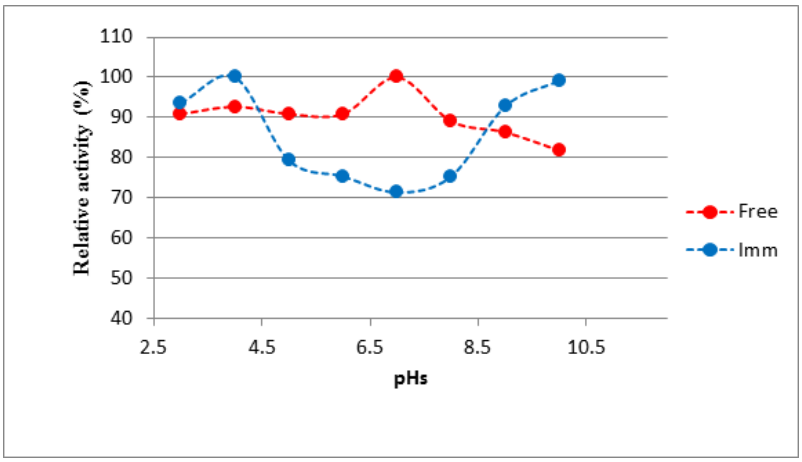

Fig. 4. pH profile of free and immobilized levansucrase.

Effect of reaction pH on free and immobilized levansucrase

The activity of both free and the immobilized levansucrase were examined by substrate dissolving at the different buffer with different pHs (3-10). Figure 4 represented the results which recorded that the optimum pH value was 7 for free levansucrase weather immobilization provide a wide range of activity The levansucrase shifted to acidic range (pH 3) and alkaline range (pH10) with 100% of activity. Previous reports, showed that levansucrase immobilization is slightly shifted the optimum pH value towards more acidic conditions. In most *Bacillus subtilis* cases [27], *Pseudomonas syringae* [28] and *Rahnella aquatilis* [29].

Effect of substrate concentration on the activity of free and immobilized levansucrase

The kinetic parameters for the free and immobilized form were calculated by Lineweaver–Burk Plot (Fig. 5). The K_m value for free and immobilized levansucrase was 2.5 and 2.85 mg/ml, respectively. An increase in K_m after enzyme immobilization could be back to the low substrate diffusion rate to the enzyme active site [19]. The increase in K_m value after immobilization was mentioned by many other investigators [30]. The free and immobilized enzyme had V_{max} values of 71.4 and 62.4 mg.min^{-1}, respectively. The lower V_{max} of the immobilized form than the free levansucrase was recorded by other authors [19].

Effect of metal ions on free and immobilized levansucrase.

The activation or inhibition effect of different metal ions and EDTA were examined. The result represented in Fig. 6 pointed clearly to the inhibitory effect of all the tested metals for free levansucrase and also dramatic inhibition was noticed with addition of metal ion to the reaction mixture of immobilized enzyme. In this finding, it was reported that heavy metal ions like Fe^{3+}, Hg^{2+} and Mn^{2+} inhibited enzyme activity significantly

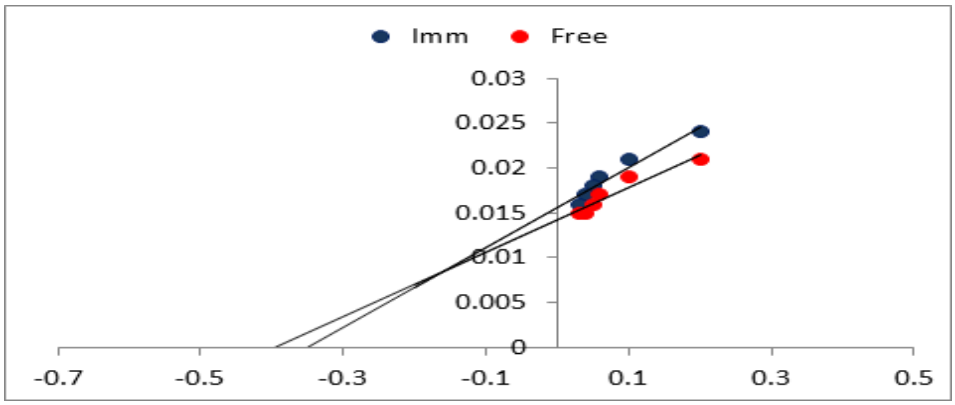

Fig. 5. Lineweaver–Burk plot for estimation of kinetic parameters for free and immobilized levansucrase.

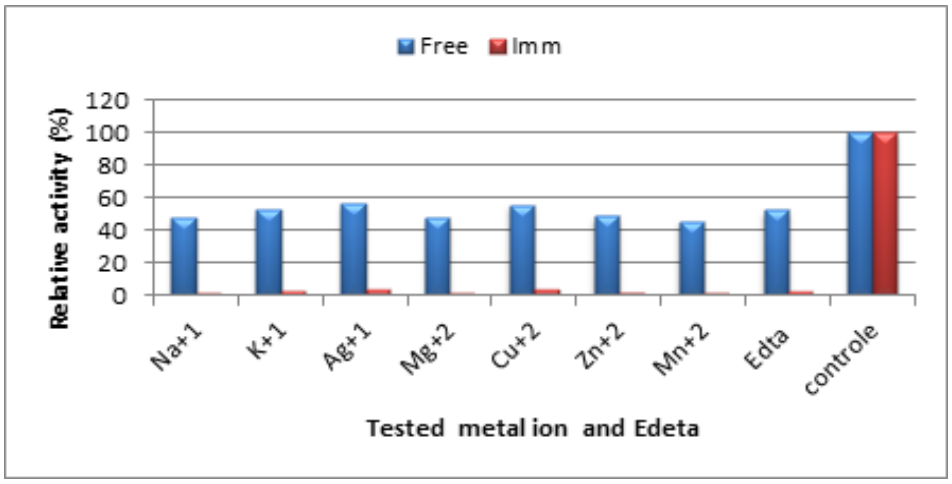

Fig. 6. Effect of addition of metal ions and EDETA on free and immobilized levansucrase.

[30]. On contrary, [32]. [31] reported that the immobilization process had protected effect on the enzyme activity from different inhibitors like $HgCl_2$ and AgCl, also surfactants such as SDS and EDTA.

Conclusion

This study introduces *Bacillus tequilensis* as novel levansucrase producers. The yielded levan was characterized by FT-IR and paper chromatography. It also, justified the use of a grafted κ-carrageenan- CMC as a suitable matrix for levansucrase immobilization by the covalent binding. A 2^2 Full factorial central composite design was done for optimizing loading time and loading activity (U/mL). Covalent immobilization increased the pH tolerance against acidic and alkaline pHs. K_m and V_{max} for both free and immobilized levansucrase were studied. An inhibitory effect of different metals ions as well as EDTA was noticed for levansucrase formulas.

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التوصيف الكيمائي لليفان مع تحسين الكبسله لانزيم الليفانسوكريز المنتج بواسطه *Bacillus tequalis*على حبيبات κ-Carrageenan –CMC

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تعتبرالسلالة البكتيرية *Bacillus tequilens* منتج جيد لإنزيم levansucrase حيث يتم انتاجه بنسبة U/ 222.2ml مع انتاج 130 جرام لكل لتر من الليفان . فى هذا البحث تم التوصيف الكيميائى لليفان المنتج بواسطة FT-IR وسجلت النتائج ان الفركتوز هو المكون الرئيسى لليفان المنتج مع نسبه قليله من الجلوكوز . تم تحميل Levansucrase التي تنتجها *Bacillus tequilens* عن طريق الارتباط التساهمي على كرجنان وكاربوكسي ميثيل السيليلوزحيث يتم تنشيطها على خطوتين :غمس الجل فى البولي ايثيلين امين ثم غلوتارالدهيد. ثم استخدام تصميم احصائىfull-factorial central composite experiment design 2^2 للوصول الى احسن الظروف لتحميل النزيم على الحبيبات الهلامية حيث تم تحميل U 14.01852/ جرام من الحبيبات وبمقارنة الأنزيم الحر والإنزيم المحمل وجد ان أظهر الإنزيم الحر درجة الحموضة المثلى عند 7 بينما زادت عملية التحميل من تحمل الإنزيم عند كلاهما مجموعة قلوية pH3 ومجموعة حمضية pH10. كان k_m بعد التحميل 85 .2 ملغم / مل مقارنة بـ 2.5 ملغم / مل من الإنزيمات الحرة. كما اصبحت السرعة القصوى min /71.4mg للإنزيم الحر بينما كانت min62.4 /mg لصيغة الإنزيم الثابتة. تم التعرف على تثبيط نشاط الإنزيم الحر منه والمقيد مع بعض أيونات الفلزات بالإضافة إلى EDTA