Carbohydrate Polymers 93 (2013) 740–746

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Production optimization of invertase by *Lactobacillus brevis* Mm-6 and its immobilization on alginate beads

Ghada E.A. Awad[a,*], Hassan Amer[a], Eman W. El-Gammal[a], Wafaa A. Helmy[a], Mona A. Esawy[a], Magdy M.M. Elnashar[b,c]

[a] Department of Chemistry of Microbial and Natural Products, National Research Center, Giza, Cairo, Egypt
[b] Centre of Excellence, Laboratories of Avanced Materials & Nanotechnology, Polymer Dept., National Research Centre, Dokki, 12311, Cairo, Egypt
[c] College of Medicine, Biochemistry Dept., Taif Uni., Taif, KSA

ARTICLE INFO

*Article history:*
Received 27 November 2012
Accepted 13 December 2012
Available online 26 December 2012

*Keywords:*
Breast milk
Invertase
Immobilization
*Lactobacillus brevis*
Central composite design
Grafted alginate beads

ABSTRACT

A sequential optimization strategy, based on statistical experimental designs, was employed to enhance the production of invertase by *Lactobacillus brevis* Mm-6 isolated from breast milk. First, a 2-level Plackett–Burman design was applied to screen the bioprocess parameters that significantly influence the invertase production. The second optimization step was performed using fractional factorial design in order to optimize the amounts of variables have the highest positive significant effect on the invertase production. A maximal enzyme activity of 1399 U/ml was more than five folds the activity obtained using the basal medium. Invertase was immobilized onto grafted alginate beads to improve the enzyme's stability. Immobilization process increased the operational temperature from 30 to 60 °C compared to the free enzyme. The reusability test proved the durability of the grafted alginate beads for 15 cycles with retention of 100% of the immobilized enzyme activity to be more convenient for industrial uses.



## 1. Introduction

Breast milk is widely acknowledged as the most complete form of nutrition for infants, with a range of benefits for infants' health, growth, immunity and development. Many studies were done on *Lactobacillus* and *Bifidobacterium* spp. isolated from breast milk and their role as probiotic. However, there are limited studies on using these bacteria in the production of important enzymes with high biotechnological approaches.

Invertase (β-D-fructofuranosid fructohydrolase, EC 3.2.1.26) catalyses the hydrolysis of sucrose into an equimolar mixture of glucose and fructose (Danisman, Tan, Kaçar, & Ergene, 2004). Inverted sugar has a lower crystallinity than sucrose, which is important in food industry to ensuring that the products remain fresh and soft for a long time. Invertase is being widely applied in food industry, particularly in the production of the chocolate with liquid center (Tomotani & Vitolo, 2007). At high concentration of sucrose this enzyme shows transglycosidase activity and thus can be used in the production of the fructooligosaccharides (FOS) – sugars with probiotic properties (Ning et al., 2010). It is worth to mention that invertase is also used in the production of artificial honey and is widely used in production of high-fructose syrup (HFS) which is largely employed as a sweetener in food and pharmaceutical industries as well as the source for attaining crystalline fructose. It has more desirable functional properties such as high osmotic pressure, high solubility, a source of instant energy as well as preventing crystallization of sugar in food products (Kurup, Subramani, Hidajat, & Ray, 2005). Moreover, invertase is used as plasticizing agents in cosmetics, medicines and paper industry.

Optimization of fermentation has long been used in enhancing the yield of many bioprocesses. Optimization studies involving a one-factor-at-a-time approach is tedious and tend to overlook the effects of interacting factors but might lead to misinterpretations of results (Zhang et al., 1996). Response surface methodology (RSM), which has been extensively applied in optimization of medium composition, conditions of enzymatic hydrolysis, and fermentation (Cui et al., 2006) is the collection of mathematical and statistical techniques for experiment design, model development, evaluation factors, and optimum conditions of different biotechnological bioprocess. Statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components. Optimization through factorial design and the application of response surface methodology is a common practice in biotechnology for optimizing the media components and process parameters (Rao, Jayaraman, & Lakshmanan, 1993; Chen, 1996).

Due to the vast use of invertase in the food industries, several attempts have already been performed to immobilize it on different supports in order to extend its stability, providing reusage possibility and preventing its loss upon processing. Many different supports have been investigated (Arica & Bayramoglu,

* Corresponding author. Tel.: +202 33335933; fax: +202 33370931.
*E-mail address:* ghadaawad18@yahoo.com (G.E.A. Awad).

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http://dx.doi.org/10.1016/j.carbpol.2012.12.039

2006; Altinok, Aksoy, Tumturk, & Hasirci, 2008). The immobilization process has been mostly realized by covalent bonding of the amino-groups of the protein to the proper functional groups of the support (Azodi, Falamaki, & Mohsenifar, 2011).

According to our knowledge, we are reporting for the first time, the ability of *Lactobacillus brevis* Mm-6 isolated from breast milk to produce invertase which can be safely used in different biotechnological approaches. A sequential optimization strategy for identified the enzyme production parameters in basal production medium as an effective tool for medium engineering. First, Plackett–Burman screening design was applied to address the most significant factors affecting enzyme production. Second, central composite design (CCD) was applied to determine the optimum level of each of the significant parameters that brings maximum invertase production. In the second part of our study, we studied the immobilization of invertase onto grafted alginate beads to improve the enzyme's stability to be convenient for different industrial applications. We optimized the enzyme loading capacity as well as examining the temperature stability and the reusability of the immobilized enzyme.

## 2. Materials and methods

### 2.1. Microorganism

#### 2.1.1. Samples collection

Breast milk samples were collected from different mothers during the first three months of breastfed. Milk samples are collected in sterile containers and store in refrigerator at 4 °C until reached to the laboratory. One ml of the sample was inoculated in tubes containing 9 ml of MRS broth medium, the inoculated tubes were incubated at 37 °C for 48 h. One milliliter of the culture was plated onto MRS agar medium, incubation was done under anaerobic condition. Successive purification was done for isolates till reaching to a single colony (Solís, de los Reyes-Gavilan, Fernández, Margolles, & Gueimonde, 2010). Bacterial isolates were routinely grown on MRS broth medium at 37 °C and preserved at −80 °C in 50% glycerol.

#### 2.1.2. Identification of invertase producing bacteria

The most potent isolate have the ability to produce invertase had been examined by cell morphology, gram staining and spore production properties. Identification of bacterial isolate was kindly carried out in City for Scientific Research, Alexandria, Egypt by using 16S rRNA tequnic.

### 2.2. Enzyme assay

Invertase activity was measured by the release of reducing sugars according to Miller (1959). The reaction mixture comprised 250 μl of 0.1 M sucrose as a substrate, 200 μl of 0.1 M acetate buffer at pH value 5.0 and 50 μl of diluted enzymatic extract. Incubation was set at 37 °C for 30 min and then the reaction was stopped by adding 1 ml of di-nitro salicylic acid (DNS), the mixture boiled for 5 min to complete color development. One invertase unit (U) was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute under assay conditions.

### 2.3. Enzyme production conditions

Cultures were allowed to grow at 37 °C in 250 ml conical flasks containing 50 ml of MRS broth medium. Then 0.5 ml ($1.2 \times 10^6$ CFU/ml) of the overnight culture was used as inoculum for the designed production medium of the following composition (g/l): Yeast extract, 2; sucrose, 100. Inoculated with 2% of ($1.2 \times 10^6$ CFU/ml) inoculum and incubated at 37 °C for 3 days. Cultures were centrifuged at 5000 rpm at 4 °C for 15 min. The culture supernatant was used as the crude enzyme. Results reported were the average values with standard deviations.

### 2.4. Statistical designs

#### 2.4.1. Plackett–Burman design

For multivariable processes such as biochemical systems, in which numerous potentially influential factors are involved, it is necessary to analyze the process with an initial screening design prior to optimization (Box, Hunter, & Hunter, 1978). Plackett–Burman experimental design (Plackett and Burman, 1946) was used to evaluate the relative importance of various nutrients for the production of invertase by *L. brevis* Mm-6 in submerged fermentation. Eleven components were selected for the study, each variable represented at two levels, high concentration (1) and low concentration (−1) in 12 trials as shown in Table 1. pH, glucose, sucrose, malt extract, peptone, yeast extract, NaCl, $CaCl_2$, $MgSO_4$, $CuSO_4$, and $FeSO_4$. Each row represented a trial run and each column represented an independent variable concentrations. Plackett–Burman experimental design is based on the first order linear model:

$$Y = B_0 + \Sigma B_i X_i \quad (1)$$

where $Y$ is the response (invertase production), $B_0$ is the model intercept and $B_i$ is the variables estimates. The effect of each variable was determined by the following equation,

$$E(X_i) = \frac{2(\Sigma M_i^+ - M_i^-)}{N} \quad (2)$$

where $E(X_i)$ is the effect of the tested variable. $M_i{}^+$ and $M_i{}^-$ represent invertase production from the trials where the variable ($X_i$) measured was present at high and low concentrations, respectively and $N$ is the number of trials in Eq. (2). The standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level ($P$-value) of each concentration effect was determined using student's $t$-test

$$t(X_i) = \frac{E(X_i)}{SE} \quad (3)$$

where $E(X_i)$ is the effect of variable $X_i$.

### 2.5. Central composite design

After the identification of components affecting the production by Plackett–Burman design three variables (malt extract, $CuSO_4$, and $FeSO_4$ concentrations) were selected for response surface methodology of central composite design (CCD). CCD proposed by (Box et al., 1978; Adinarayana, Ellaiah, Srinivasulu, Bhavani, & Adinarayana, 2003) was selected for this study, a $2^3$ factorial design with six star points and six replicates at the central points were employed to fit the second-order polynomial model, the experimental design consisted of 20 runs and the independent variables were studied at five different levels. The experimental design used for the study is shown in Table 3. All the experiments were done in triplicate and the average of invertase production obtained was taken as the dependent variable or response ($Y$). The second-order polynomial coefficients were calculated and analyzed using the 'SPSS' software (Version 16.0) second degree polynomials, Eq. (4), which included all interaction terms, were used to calculate the predicted response:

$$Y_{Activity} = \beta_0 + \beta_1 \times 1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_2^2 X_2^2 + \beta_3^3 C_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

where $Y_{Activity}$ was the predicted production of invertase (U/ml), and $X_1$, $X_2$ and $X_3$ were the independent variables corresponding

**Table 1**
Randomized Plackett–Burman experimental design showed coded and uncoded variables levels for evaluating its influencing on invertase production from *Lactobacillus brevis* Mm-6.

| Run no. | pH $X_1$ | Malt extract $X_2$ | Peptone $X_3$ | Yeast extract $X_4$ | Sucrose $X_5$ | Glucose $X_6$ | NaCl $X_7$ | $CaCl_2$ $X_8$ | $MgSO_4$ $X_9$ | $CuSO_4$ $X_{10}$ | $FeSO_4$ $X_{11}$ | Invertase activity (U/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1(6) | 1(2) | −1(0.5) | 1(2) | −1(50) | 1(100) | −1(0.1) | 1(1.0) | −1(0.01) | 1(0.01) | −1(0.001) | 670.1 ± 0.74 |
| 2 | 1(8) | 1(2) | −1(0.5) | −1(0.5) | −1(50) | 1(100) | −1(0.1) | −1(0.1) | −1(0.01) | 1(0.01) | 1(0.01) | 860.5 ± 0.32 |
| 3 | 1(8) | 1(2) | 1(2) | −1(0.5) | −1(50) | −1(50) | −1(0.1) | −1(0.1) | 1(0.1) | 1(0.01) | 1(0.01) | 1200 ± 0.74 |
| 4 | 1(8) | −1(0.5) | 1(2) | −1(0.5) | 1(100) | 1(100) | 1(1.0) | −1(0.1) | 1(0.1) | −1(0.001) | 1(0.01) | 466.8 ± 1.2 |
| 5 | 1(6) | 1(2) | 1(2) | 1(2) | 1(100) | 1(100) | 1(1.0) | 1(1.0) | 1(0.1) | 1(0.01) | −1(0.001) | 628.2 ± 1.14 |
| 6 | 1(8) | 1(2) | −1(0.5) | 1(2) | 1(100) | −1(50) | 1(1.0) | 1(1.0) | −1(0.01) | 1(0.01) | 1(0.01) | 709.9 ± 0.22 |
| 7 | 1(8) | −1(0.5) | 1(2) | 1(2) | −1(50) | 1(100) | −1(0.1) | 1(1.0) | 1(0.1) | −1(0.001) | 1(0.01) | 639.9 ± 0.21 |
| 8 | −1(6) | 1(2) | 1(2) | −1(0.5) | 1(100) | −1(50) | 1(1.0) | −1(0.1) | 1(0.1) | 1(0.01) | −1(0.001) | 833.6 ± 0.74 |
| 9 | 1(8) | −1(0.5) | −1(0.5) | 1(2) | 1(100) | −1(50) | 1(1.0) | 1(1.0) | −1(0.01) | −1(0.001) | 1(0.01) | 656.1 ± 1.12 |
| 10 | −1(6) | −1(0.5) | 1(2) | 1(2) | −1(50) | −1(50) | −1(0.1) | 1(1.0) | 1(0.1) | −1(0.001) | −1(0.001) | 593.8 ± 1.14 |
| 11 | −1(6) | −1(0.5) | −1(0.5) | −1(0.5) | 1(100) | 1(100) | 1(1.0) | −1(0.1) | −1(0.01) | −1(0.001) | −1(0.001) | 747.5 ± 0.72 |
| 12 | −1(6) | −1(0.5) | −1(0.5) | −1(0.5) | −1(50) | −1(50) | −1(0.1) | −1(0.1) | −1(0.01) | −1(0.001) | −1(0.001) | 233.0 ± 0.55 |

Numbers between bracket represent actual concentrations of tested variables (g/l); $R^2$ = 99.99%.

to the concentration of malt extract, $CuSO_4$ and $FeSO_4$ respectively; $\beta_0$ was the intercept, $\beta_1$, $\beta_2$, $\beta_3$ were linear coefficients, $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ are quadratic coefficients, $\beta_{12}$, $\beta_{13}$, $\beta_{23}$ are cross product coefficients. 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 coefficient of determination for each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

### 2.6. Immobilization of invertase onto grafted alginate beads

#### 2.6.1. Preparation of grafted alginate beads

Alginate beads were made by using the Encapsulator, model IR50 purchased from Innotech Encapsulator (Switzerland). Alginate was dissolved in distilled water to give a final concentration of 1.5% (w/v) and dropped through 300 μm nozzle in a hardening solution using the two-step method (Danial, Elnashar, & Awad, 2010), the beads were treated by 2% (w/v) $CaCl_2$ ($Ca^{2+}$) for 2 h then dropped in a solution of 2% (w/v) $CaCl_2$ dissolved in 4% (v/v) polyethylenimine at pH 8 for 2 h. The treated gels were then soaked in a solution of 2.5% (v/v) glutaraldehyde (GA) for 2 h to incorporate the new functionality, aldehyde group.

#### 2.6.2. Immobilization of invertase onto grafted alginate beads

The immobilization process is done by soaking 0.5 g of the gel beads in 5 ml of invertase (appropriate dilution was done in 0.1 M acetate–phosphate buffer at pH 5 for 24 h). The gel beads were washed twice thoroughly for 30 min with acetate buffer to get rid of any unbound enzyme. The beads containing the immobilized enzyme were stored at 4 °C for further measurements. For determination of activity of the immobilized enzyme, an equal amount of 0.5 g gel of gel beads were incubated into 1.25 ml of 0.1 M sucrose and 1 ml of buffer for 30 min at 37 °C, an aliquot from the reaction mixture was assayed for fructose determination as described before in material and methods.

**Table 2**
Statistical analysis of Plackett–Burman design showing coefficient values, *t*-and *P*-values for each variable on invertase activity.

| Variables | Coefficient | *t*-Statistics | *P*-value | Confidence level (%) |
|---|---|---|---|---|
| Intercept | 686.617 | | | |
| pH | 44.250 | 1.317 | 0.108 | 89.14 |
| Malt extract | 261.33 | 3.912 | 0.001 | 99.85 |
| Peptone | −6.467 | −1.329 | 0.137 | 89.54 |
| Yeast extract | 94.400 | −0.733 | 0.240 | 76.00 |
| Sucrose | 44.733 | 2.065 | 0.032 | 96.71 |
| Glucose | −17.783 | −0.528 | 0.304 | 69.58 |
| NaCl | −12.933 | −1.152 | 0.137 | 86.18 |
| $CaCl_2$ | −36.950 | 1.212 | 0.126 | 87.34 |
| $MgSO_4$ | 40.433 | −1.329 | 0.137 | 86.21 |
| $CuSO_4$ | 130.433 | 3.138 | 0.005 | 99.47 |
| $Fe_2SO_4$ | 68.917 | 2.829 | 0.008 | 99.18 |

### 2.7. Optimum temperature

The optimum temperature for the free and immobilized invertase was examined by incubating the reaction mixture for both free and immobilized enzymes at different temperatures ranging from 25 to 90 °C for 30 min. The optimum temperature has been taken as 100% activity and the relative activity at each temperature was expressed as a percentage of the 100% activity.

### 2.8. Operational stability

The reusability of immobilized invertase on alginate beads was studied by incubation of 0.5 g of loaded gel beads with 1.25 ml of 0.1 M sucrose and 1 ml of buffer for 30 min at 60 °C. Fructose was assayed as described before. The same gel beads were then washed with acetate buffer and re-incubated with another substrate solution; this procedure was repeated 20 times, and the initial activity was considered as 100%. The relative activity was expressed as a percentage of the starting operational activity.

## 3. Results and discussion

### 3.1. Identification of the invertase producing isolate

Among 30 *Lactobacillus* spp. isolated from breast milk samples, isolate No. 6. gives a highest yield of invertase was chosen for further investigation.

Isolate No. 6 was characterized as Gram-positive, nonspore forming rods and catalase-negative. The invertase producing bacterial isolate was identified by using 16S rRNA tequnic in City for Scientific Research, Alexandria, Egypt as *L. brevis* and designated as *L. brevis* Mm-6. *Lactobacillus* spp. *lactobacilli, staphylococci, streptococci, micrococci*, and *enterococci* are the bacterial strains which commonly isolated from breast milk, which should be considered as components of the natural microflora rather than as mere contaminant bacteria (Wold & Adlerberth, 2000; Martín et al., 2005).

### 3.2. Optimization of invertase production by multi-factorial experiments

A sequential optimization approaches were applied in this study. The first approach deals with screening for nutritional

**Table 3**
Central composite design (CCD) consisting of 20 experiments for three experimental factors in coded and actual values for the production of invertase by *Lactobacillus brevis* Mm-6.

| Trial no. | Factor levels | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Malt extract ($X_1$) | | CuSO4 ($X_2$) | | FeSO4 ($X_3$) | | Invertase activity (U/ml) | |
| | Coded | Actual(g/L) | Coded | Actual (g/l) | Coded | Actual (g/l) | Observed | Predicted |
| 1[a] | −1 | 1 | −1 | 0.005 | −1 | 0.005 | 250 ± 1.3 | 406 |
| 2[a] | +1 | 4 | −1 | 0.005 | −1 | 0.005 | 1036 ± 1.4 | 1077 |
| 3[a] | −1 | 1 | +1 | 0.02 | −1 | 0.005 | 959 ± 0.74 | 701 |
| 4[a] | +1 | 4 | +1 | 0.02 | −1 | 0.005 | 1300 ± 1.2 | 1374 |
| 5[a] | −1 | 1 | −1 | 0.005 | +1 | 0.02 | 1359 ± 1.2 | 1212 |
| 6[a] | +1 | 4 | −1 | 0.005 | +1 | 0.02 | 907 ± 3.2 | 1032 |
| 7[a] | −1 | 1 | +1 | 0.02 | +1 | 0.02 | 881 ± 3.4 | 861 |
| 8[a] | +1 | 4 | +1 | 0.02 | +1 | 0.02 | 803 ± 0.76 | 675 |
| 9[b] | −2 | 0.5 | 0 | 0.01 | 0 | 0.01 | 725 ± 0.99 | 936 |
| 10[b] | +2 | 6 | 0 | 0.01 | 0 | 0.01 | 1062 ± 1.3 | 1020 |
| 11[b] | 0 | 2 | −2 | 0.001 | 0 | 0.01 | 1026 ± 3.4 | 819 |
| 12[b] | 0 | 2 | +2 | 0.04 | 0 | 0.01 | 120 ± 2.2 | 230 |
| 13[b] | 0 | 2 | 0 | 0.01 | −2 | 0.001 | 767 ± 1.4 | 691 |
| 14[b] | 0 | 2 | 0 | 0.01 | +2 | 0.04 | 480 ± 1.4 | 436 |
| 15[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1399 ± 3.5 | 1411 |
| 16[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1392 ± 1.3 | 1411 |
| 17[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1396 ± 0.72 | 1411 |
| 18[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1398 ± 1.1 | 1411 |
| 19[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1399 ± 2.4 | 1411 |
| 20[c] | 0 | 2 | 0 | 0.01 | 0 | 0.01 | 1394 ± 3.2 | 1411 |

[a] Fractional 23 factorial design.
[b] Star points.
[c] Central points.

factors affecting growth of *L. brevis* Mm-6 with respect to invertase production. The second approach is to optimize the factors that control the enzyme production process.

### 3.2.1. Evaluation of the factors affecting invertase productivity

In the first approach, the Plackett–Burman design was applied to reflect the relative importance of various medium components. Eleven different factors (variables) including medium constitution and initial pH were chosen to perform this optimization process. The averages of invertase activity for the different trials were given in U/ml (Table 1). The main effect of each variable upon invertase activity was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (−1) of that factor. The data in Table 1 showed a wide variation from 233 to 1200 U/ml of invertase activity. This variation reflects the importance of medium optimization to attain higher productivity. The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. The main effects of the examined factors on the enzyme activity were calculated and presented graphically (Fig. 1) offers the view for the ranking of factor estimates obtained by Plackett–Burman design.

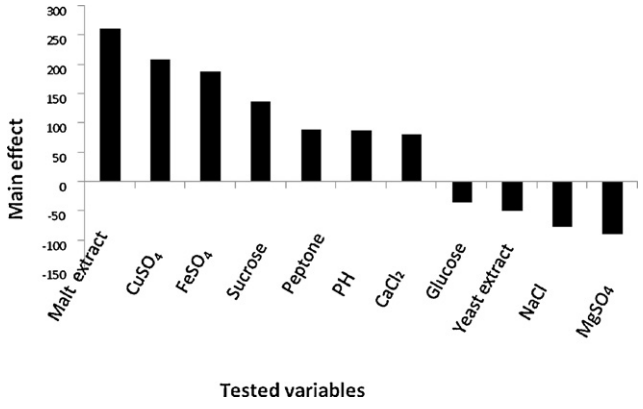


**Fig. 1.** Effect of medium composition factors on the enzyme activity (U/ml) produced by *Lactobacillus brevis* Mm-6.

Table 2. shows the regression coefficients, calculated *t-test*, *P-values* of the tested variables. Malt extract, $CuSO_4$, $FeSO_4$, sucrose, peptone, culture pH and $CaCl_2$, showed positive effect on invertase activity. Glucose, yeast extract, NaCl and $MgSO_4$were contributed negatively.

The first order linear model describing the correlation between the eleven factors and the Invertase activity could be presented as follows:

$$Y_{activity} = 686.617 + 44.250X_1 + 104.583X_2 - 6.467X_3 + 94.400X_4 + 44.733X_5 - 17.783X_6 - 12.933X_7 - 36.950X_8 + 40.433X_9 + 130.433X_{10} + 68.917X_{11} \quad (5)$$

Based on the calculated *t*-test and *P*-values (Table 2), it was evident that the medium components malt extract, $CuSO_4$ and $FeSO_4$ were found to be the most significant variables affecting invertase productivity. Effect of malt extract on invertase production was referred to its importance as a complex of nitrogen source. It is reported that nitrogen source is essential for microbial growth of *Lactobacillus* spp. (Kwon, Cheon Lee, Gyo Lee, Keun, & Chang, 2000). Copper is an essential trace element in living systems, where it serves as a cofactor in enzymes that function in energy generation, oxygen transport, signal transduction and many other processes (Kim, Nevitt, & Thiele, 2008; Claus, 2010; Schut, Zauner, Hampel, Konig, & Claus, 2011). Other variables with less significant effect were not included in the next optimization experiment, but instead were used in all trials at their (+1) level, for the positively contributing variables. According to these results, a medium of the following composition (g/l), sucrose, 100; peptone, 2; $CaCl_2$, 1 and pH 8 was used as a plain medium for further investigations.

### 3.2.2. Optimization of the culture conditions by central composite design

In order to search for the optimum concentration of the most significant medium components (malt extract, $CuSO_4$ and $FeSO_4$)

**Table 4**
Model coefficients estimated by multiples linear regression (significance of regression coefficients).

| Term | Regression coefficient | Standard error | *t*-Test | *P*-value |
|---|---|---|---|---|
| Intercept | −1513 | 357.820 | −4.228 | 0.002 |
| $X_1$ | 695 | 146.675 | 4.741 | 0.001 |
| $X_2$ | 142,561 | 27180.033 | 5.245 | 0.000 |
| $X_3$ | 193,847 | 27180.033 | 7.132 | 0.000 |
| $X_1{}^2$ | −75.334 | 17.441 | −4.319 | 0.002 |
| $X_2{}^2$ | − 4.269 | 644166.361 | −6.684 | 0.000 |
| $X_3{}^2$ | − 4.306 | 644166.361 | −6.628 | 0.000 |
| $X_1X_2$ | −149.125 | 4899.989 | −0.030 | 0.976 |
| $X_1X_3$ | −18896 | 4899.989 | −3.856 | 0.003 |
| $X_2X_3$ | −2.906 | 975943.688 | −2.978 | 0.014 |

*F* value = 11.591; $P > F = 0.0001$; $R^2 = 0.913$; $R = 0.955$; adjusted $R^2 = .834$.

showing confidence level 99% and above in the Plackett–Burman design for invertase production, experiments were performed according to the CCD experimental. The coded and uncoded levels of the three independent variables investigated was listed in Table 3. The table also showed the CCD experimental plan, the observed and predicted invertase production. Multiple regression analysis of the experimental data gave the following second order polynomial equation (6).

$$Y_{activity} = -1513.029 + 695.457X_1 + 142561X_2 + 193847X_3 - 75.334X_1^2 - 4.269X_2^2 - 4.306X_3^2 - 149.125X_1X_2 - 18896X_1X_3 - 2.906X_2X_3 \quad (6)$$

where $Y_{activity}$ is the response (invertase production) and $X_1, X_2$ and $X_3$ are the coded values of the test variables (malt extract, $CuSO_4$and $FeSO_4$) respectively. The three-dimensional response surface and the two-dimensional contour plots are the graphical representations of the regression equation. They are helpful in understanding both the main and the interaction effects of the factors on the response value. Fig. 2A–C shows the response surface and contour plots of malt extract and $CuSO_4$, malt extract and $FeSO_4$and $CuSO_4$and $FeSO_4$on invertase production respectively, keeping the other component at the fixed zero level.

Table 4 showed the regression results from the data of central composite designed experiments. The larger the magnitude of the *t*-value and smaller the *P*-value, the more significant is the corresponding coefficient (Aravindan & Viruthagiri, 2007). This implies that the variable with the largest effect was the linear effect of the $FeSO_4$ concentration and the squared term of the $FeSO_4$concentration. This importance of $FeSO_4$ as a medium component in the invertase production was not clear in the literatures. Furthermore, quadratic effect of the $FeSO_4$ and $CuSO_4$ are more significant than the malt extract.

The results obtained by (ANOVA) analysis showed a significant *F*-value (11.591) which implied the model to be significant. Model terms having values of Prob > *F* (0.0001) less than 0.05, considered significant. The determination of coefficient ($R^2$) was calculated as 0.913 for invertase activity (a value of $R^2 > 0.75$ indicated the aptness of the model) which indicated the statistical model could explain 91.3% of variability in the response. The goodness of the model can be checked by the determination of coefficient ($R^2$) and correlation coefficient ($R$). The $R^2$ value is always between 0 and 1. The closer the $R^2$ to 1, the stronger the model and the better in predicted response (Munk, Pásková, & Hanus, 1963). The value of $R$ (0.955) for (Eq. (6)) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. An overall 5.14-fold increase in invertase was being achieved after application of RSM. This reflects the necessity and value of optimization process. There is no report available on optimization of invertase by using RSM production of invertase. All reports available so far are on optimization of invertase by one variable at a time by using *Saccharomyces cerevisiae* (Andjelkovic, Pićuric, & Vujčic, 2010) But in general different studies were done for optimization of enzymes production using RSM (Abdel-Fattah, Saeed, Gohar, & El-Baz, 2005; Awad, Elnashar, & Danial, 2011a; Awad et al., 2011b).

### 3.3. Validation of the model

The validation was carried out under optimum conditions of the media predicted by the polynomial model. The experimental invertase production of 1399 U/ml was obtained which is closer to the predicted invertase production of 1411 U/ml after 3 days of fermentation validating the proposed model. The production of invertase by *L. brevis* Mm-6 at the optimized conditions is much higher than that of invertase produced by *Yarrowia lipolytica SUC+ transformants* and reported by Lazar, Walczak, & Robak (2011). The combination of Plackett–Burman design and central composite design was shown to be effective and reliable in selecting the statistically significant factors and finding the optimal concentrations of those factors for invertase production by *L. brevis* Mm-6 in submerged fermentation. A second order polynomial model was established using central composite design to identify the relationship between the three factors and the invertase yield. The final concentrations of the medium components optimized with RSM were (g/l) malt extract, 2; peptone, 2; sucrose, 100; $CaCl_2$, 1; $CuSO_4$, 0.01 and $FeSO_4$, 0.01. The initial pH was adjusted to 8 as a plane medium for further investigations.

### 3.4. Immobilization of invertase onto grafted alginate beads

#### 3.4.1. Optimization of the enzyme loading capacity

Optimization of enzyme loading capacity (ELC) was carried out by soaking 0.5 g of the gel beads in 5 ml of original enzyme solution and diluted enzyme 1:20, 1:15, 1:10, 1:05 and 1:02 in 0.1 M acetate phosphate buffer at pH 5 for 24 h. The ELC increased gradually by increasing the enzyme concentration, to reach ELC of 825 U/g gel beads at 1:05 dilution, after which any increase of the enzyme concentration has slight effect on the ELC to reach its maximum ELC. of 850 U/g gel beads using the original crude enzyme (data not shown). That could be explained that most of aldehyde groups have been engaged with the enzymes at 1: 05 dilution and any more enzymes have less chances to find free aldehyde groups to bind with Elnashar, Danial, & Awad (2009). This result is in agreement with the results obtained in our previous study on the same polymer by Elnashar and Yassin (2009a) and Danial et al. (2010)

#### 3.4.2. Optimization of temperature of immobilized invertase on grafted alginate

One of the main goals of this article was to improve the enzyme's operational temperature to be suitable for industrial use. The operational temperature of the enzyme covalently immobilized to the grafted gel revealed higher temperature stability over the free enzyme, as shown in Fig. 3.where the optimum temperature of the free enzyme was at 30 °C, and that for the immobilized enzyme was at 60 °C. The shift of the optimum temperature toward higher temperatures when the biocatalyst was immobilized indicated that the enzyme structure is strengthened by the immobilization process. This could be regarded to the formation of a molecular cage around the protein molecule (enzyme) to protect the enzyme from the high temperature of the bulk solution (Tor, Dror, & Freeman, 1989; Elnashar & Yassin (2009b)).

#### 3.4.3. Operational stability of immobilized invertase

The main advantage of immobilization of enzyme is the easy separation and reusability of the enzyme. The results indicated that

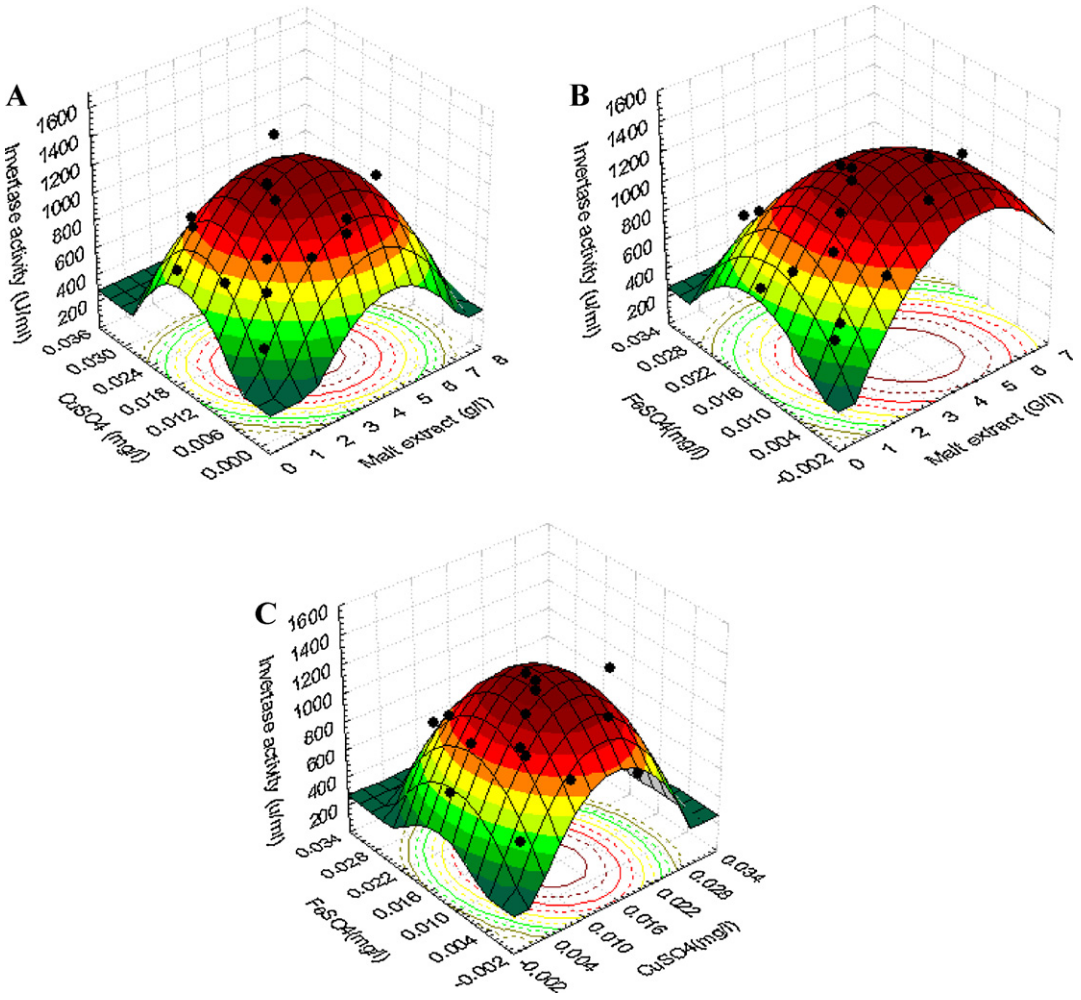


**Fig. 2.** (**A**) Response surface plot of invertase production by *Lactobacillus brevis* Mm-6 showing the interactive effects of different concentrations of malt extract and $CuSO_4$ at $X_3 = 0$. (**B**) Response surface plot of invertase production by *Lactobacillus brevis* Mm-6 showing the interactive effects of different concentrations of malt extract and $FeSO_4$ at $X_2 = 0$. (**C**) Response surface plot of invertase production by *Lactobacillus brevis* Mm-6 showing the interactive effects of different concentrations of $CuSO_4$ and $FeSO_4$ at $X_1 = 0$.

the immobilized invertase using the grafted alginate retained 100% of its activity after 15 reuses and then it started to decline to reach around 80% after 20 cycles (Fig. 4). These results was for the favor of grafted alginate as it uses the covalent technique. In another word, the immobilized invertase covalently immobilized to the grafted alginate could be reused more times than other techniques using physical bonds. For example, Tanaka, Kurosawa, Kokufuta, and Veliky (1984) used polyethylene amine to entrap the glucoamylase into alginate, and they retained only 60% of the enzyme activity by the 8th use, whereas we retained 100% of the immobilized enzyme activity for 15th use. These results confirmed the superiority of covalent technique in solving the enzyme loss problem through alginate pores. According to the previous results, the grafted alginate immobilization recommended to be used in industrial application as it saves time, enzymes and carriers consumed. However, the slight decrease in enzyme activity by the 20th use

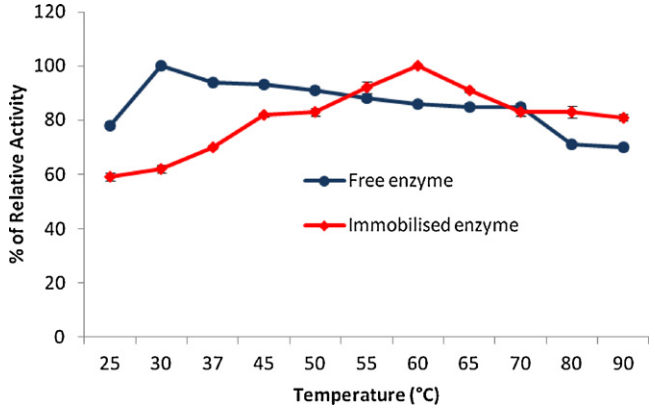


**Fig. 3.** Temperature–activity profile for immobilized and free invertase.

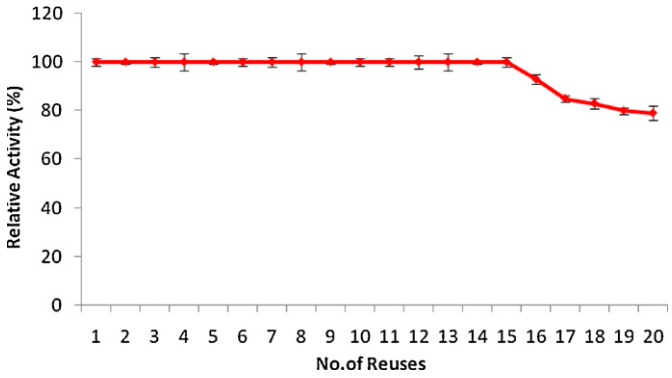


**Fig. 4.** Operational stability of immobilized invertase on and grafted alginate.

using the grafted gel could be attributed to inactivation of enzyme due to continuous use (Nakane, Ogihara, Ogata, & Kurokawa, 2001).

## 4. Conclusions

This study, focused in invertase production from *L. brevis* Mm-6 milk breast isolate. According to our knowledge, this is the first study of this strain in invertase production. The optimization of the enzyme production by using statistical experimental design was done. A highly significant quadratic polynomial equation obtained by the central composite design was important for determining the optimal concentrations of constituents that have significant effects on invertase production. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the invertase production process. The immobilization process revealed a high enzyme loading capacity of 850 U/g gel beads with an optimum thermal stability at 60 °C compared to the free enzyme, which was at 30 °C. Moreover, the immobilized enzyme has been reused for 15 times without any loss of its activity. As a result, the new immobilized enzyme has proven to be a good candidate in the industries.

## Acknowledgements

This work was supported by National Research Center, Chemistry of Natural and Microbial Products Department and the Center of Excellence for Advanced Sciences, Group of Biopolymers & Nanobiotechnology in Egypt.

## References

Abdel-Fattah, Y., Saeed, H. M., Gohar, Y. M., & El-Baz, M. A. (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochemistry*, *40*, 1707–1711.

Adinarayana, K., Ellaiah, P., Srinivasulu, B., Bhavani, R., & Adinarayana, G. (2003). Response surface methodological approach to optimize the nutritional parameters for neomycin production by *Streptomyces marinensis* under solid-state fermentation. *Process Biochemistry*, *38*, 1565–1572.

Altinok, H., Aksoy, S., Tumturk, H., & Hasirci, N. (2008). Covalent immobilization of invertase on chemically activated poly (styrene-2-hydroxyethyl methacrylate) microbeads. *Journal Food Biochemistry*, *32*, 299–315.

Andjelkovic, U., Pićuric, S., & Vujčic, Z. (2010). Purification and characterisation of *Saccharomyces cerevisiae* external invertase isoforms. *Food Chemistry*, *120*, 799–804.

Aravindan, R., & Viruthagiri, T. (2007). Sequential optimization of culture medium for extracellular lipase production by *Bacillus spharicus* using statistical methods. *Journal Chemical Technology Biotechnology*, *8*, 460–470.

Arica, M. Y., & Bayramoglu, G. (2006). Invertase reversibly immobilized onto polyethylenimine-grafted poly(GMA-MMA) beads for sucrose hydrolysis. *Journal Molecular Catalysis B: Enzymes*, *38*, 131–138.

Awad, G. E. A., Elnashar, M. M. M., & Danial, E. N. (2011 a). Optimization of phytase production by *Penicillium funiculosum NRC467* under solid state fermentation by using full factorial design. *World Applied Science Journal*, *15*, 1635–1644.

Awad, G. E. A., Salam, W. A., El-diwany, A., Salama, B. A., Abdelkader, A. F., & Esawy, M. A. (2011). Keratinase production by *Bacillus pumilus* GHD in solid-state fermentation using sugar cane bagasse: optimization of culture conditions using a Box–Behnken experimental design. *Annals of Microbiology*, *61*, 663–672.

Azodi, M., Falamaki, C., & Mohsenifar, A. (2011). Sucrose hydrolysis by invertase immobilized on functionalized porous silicon. *Journal Molecular Catalysis B: Enzymes*, *69*, 154–216.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experiments*. New York: Wile., pp. 291–334.

Chen, H. C. (1996). Optimizing the concentrations of carbon, nitrogen and phosphorus in citric acid fermentation with response surface method. *Food Biotechnology*, *10*, 13–27.

Claus, H. (2010). Copper-containing oxidases: occurrence in soil microorganisms, properties and applications. In I. Sheremati, & A. Varma (Eds.), *Soil biology. Soil heavy metals* (pp. 281–314). Heidelberg: Springer Verlag.

Cui, F. J., Li, Y., Xu, Z. H., Xu, H. Y., Sun, K., & Tao, W. Y. (2006). Optimization of the medium composition for production of mycelial biomass and exo-polymer by *Grifola frondosa* GF9801 using response surface methodology. *Bioresource Technology*, *97*, 1209–2121.

Danial, E. N., Elnashar, M. M. M., & Awad, G. E. A. (2010). Immobilized inulinase on grafted alginate beads prepared by the one-step and the two-steps methods. *Industrial Engineering Chemistry Researches*, *49*, 3120–3125.

Danisman, T., Tan, S., Kaçar, Y., & Ergene, A. (2004). Covalent immobilization of invertase on microporous pHEMA-GMA membrane. *Food Chemistry*, *85*, 461–466.

Elnashar, M. M. M., Danial, E. N., & Awad, G. E. A. (2009). Novel carrier of grafted alginate for covalent immobilization of inulinase. *Industrial Engineering Chemistry Researches*, *48*, 9781–9785.

Elnashar, M. M. M., & Yassin, A. M. (2009a). Covalent immobilization of β-galactosidase on carrageenan coated chitosan. *Applied Polymer Science*, *114*, 17–24.

Elnashar, M. M. M., & Yassin, A. M. (2009b). Lactose hydrolysis by β-galactosidase covalently immobilized to thermally stable biopolymers. *Applied Biochemistry Biotechnology*, *159*, 426–437.

Kim, B. E., Nevitt, T., & Thiele, D. J. (2008). Mechanisms for copper acquisition, distribution and regulation. *Nature chemical Biology*, *4*, 176–185.

Kurup, A. S., Subramani, H. J., Hidajat, K., & Ray, A. K. (2005). Optimal design and operation of SMB bioreactor for sucrose inversion. *Chemical Engineering Journal*, *108*, 19–33.

Kwon, S., Cheon Lee, P., Gyo Lee, E., Keun, Y., & Chang, N. (2000). Production of lactic acid by *Lactobacillus rhamnosus* with vitamin-supplemented soybean hydrolysate. *Enzyme and Microbial Technology*, *26*, 209–215.

Lazar, Z., Walczak, E., & Robak, M. (2011). Simultaneous production of citric acid and invertase by *Yarrowia lipolytica* SUC+ transformants. *Bioresource Technology*, *102*, 6982–6989.

Martín, R., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., & Rodríguez, J. M. (2005). Probiotic potential of 3 lactobacilli strains isolated from breast milk. *Journal of Human Lactation*, *21*, 18-17.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Annal Chemistry*, *31*, 42.

Munk, V., Pásková, J., & Hanus, J. (1963). Glucose oxidase of *Aspergillus niger*. *Folia Microbiologica*, *8*, 203–214.

Nakane, K., Ogihara, T., Ogata, N., & Kurokawa, Y. (2001). Entrap-immobilization of invertase on composite gel fiber of cellulose acetate and zirconium alkoxide by sol–gel process. *Journal of Applied Polymer Science*, *81*, 2084.

Ning, Y., Wang, J., Chen, J., Yang, N., Jin, Z., & Xu, X. (2010). Production of neofructooligosacharides using free-whole-cell biotransformation by *Xanthophyllomyces dendrorhous*. *Bioresource Technology*, *101*, 7472–7478.

Plackett, R. l., & Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika*, *37*, 305–325.

Rao, P. V., Jayaraman, K., & Lakshmanan, C. M. (1993). Production of lipase by *Candida rugosa* in solid-state fermentation, 2. Medium optimization and effect of aeration. *Process Biochemistry*, *28*, 391–395.

Schut, S., Zauner, S., Hampel, G., Konig, H., & Claus, H. (2011). Biosorption of copper by relevant lactobacilli. *International Journal of Food Microbiology*, *145*, 126–131.

Solís, G., de los Reyes-Gavilan, C. G., Fernández, N., Margolles, A., & Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe*, *16*, 307–310.

Tanaka, H., Kurosawa, H., Kokufuta, E., & Veliky, I. (1984). Preparation of immobilized glucomylase using ca-alginate gelcoated with partially quaterized polyethleneimine. *Biotechnology Bioengineering*, *26*, 1393.

Tomotani, E. J., & Vitolo, M. (2007). Production of high-fructose syrup using immobilized invertase in a membrane reactor. *Journal of Food Engineering*, *80*, 662–667.

Tor, R., Dror, Y., & Freeman, A. (1989). Enzyme stabilization by bilayer encagement. *Enzyme Microbial Technology*, *1*, 74.

Wold, A. E., & Adlerberth, I. (2000). Breast feeding and the intestinal microflora of the infant—implications for protection against infectious diseases. *Advances in Experimental Medicinal and Biology*, *478*, 77–93.

Zhang, J., Marcin, C., Shifflet, M. A., Salmon, P., Brix, T., Greasham, R., Buokland, B., & Chartrain, M. (1996). Development of a defined medium fermentation process for physotigmine production by *Streptomyces griseofuscus*. *Applied Microbial and Biotechnology*, *44*, 568–575.