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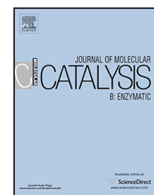

# Enhancement of the hyaluronidase production from isolated *Staphylococcus aureus* using factorial design technique and partial purification

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ABSTRACT

Hyaluronidase is an enzyme has a great potential in diverse medical fields. A group of bacterial isolates was screened for the production of hyaluronidase. The most potent isolate was identified depending on its morphological, biochemical and molecular characteristics. Thereafter, the new powerful enzyme producer was identified as *Staphylococcus aureus*. Optimization strategy was conducted to enhance the enzyme production by *S. aureus* in a medium containing a waste material (milk whey). The first step of the optimization process included choosing of the most significant factors after screening with Plackett–Burman model. Eleven factors, medium composition and some cultural conditions, were screened. Hence, the most significant factors were subjected to further optimization step using Box–Behnken model. Box–Behnken model had the ability to suggest the optimum concentrations of the significant parameters and to predict the maximal enzyme activity. The predicted enzyme activity was 500 U/ml. The predicted value was verified experimentally, and the maximum enzyme activity reached 492 U/ml. Overall, the final enzyme outcome 492 U/ml is considered absolutely high if it compared with all previous findings with free cells. In addition to using of competitive cheaper media, attempts were made to increase the effectiveness of the enzyme through partial purification.

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## 1. Introduction

Hyaluronidase is able to cleave hyaluronon within the tissues and this will cause a considerable increase in membrane permeability. The reduced viscosity can facilitate the diffusion of the injected fluids within the tissues. This will increase the absorption and dispersion of many injected drugs such as antibiotics and many other drugs. Moreover, hyaluronidase promotes resumption of excess fluid and improves the effectiveness of local anesthesia [1].

Because of these worthy activities, hyaluronidase was widely used in many medical fields like orthopedics, surgery, dentistry [2], ophthalmology [3] internal medicine, oncology [4] dermatology and gynecology [5].

These medical, physiological, biological and commercial applications of hyaluronidase reveal the requirement for continuous improvement of the production process. Sahoo et al. [6] improved the hyaluronidase production by *Streptococcus EQUI* SED9 and the highest yield was 250 μ/ml. Zoharb et al. [7] recorded that the amount of the produced enzyme was 8.04 U/ml. Mahesh [8] utilized immobilized cells of *Streptococcus mitis* and the 591 μ/ml were produced. Kotra et al. [9] achieved 1368 mg/l of hyaluronidase using *Streptococcus zooepidemicus*.

The current work focused on selection of producer strain after screening of some bacterial isolates for their abilities to produce hyaluronidase. These bacteria were isolated from soil samples and clinical specimens obtained from different sources in Cairo (Egypt) and Najran region (KSA).

After identification of the most potent strain the investigation was mainly devoted to maximize the amounts of the produced enzyme using factorial design technique. The optimization strategy includes selection of the most significant effective factors using Plackett–Burman Model. The Plackett–Burman model is usually used for screening a large number of factors to select the most significant ones [6,10]. Consequently, Box–Behnken model was utilized to detect the optimal and to predict the maximum enzyme outputs. To the best of our knowledge, this was the first time for such optimization strategy to be applied to enhance hyaluronidase production. In addition to the optimization strategy, fractionation of the culture filtrate was conducted to increase the efficiency of the enzyme after removing of many contaminant proteins. Fractional

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precipitation is used to increase the specific activity of the enzyme through separation of the impurities. Isolation of the enzyme by fractional precipitation was achieved by acetone, ethanol and ammonium sulfate.

## 2. Materials and methods

### 2.1. Microorganisms

All strains that are used throughout this work were isolated from clinical specimens and soil samples obtained from different places in Egypt (Cairo) and Najran (KSA). The isolated bacterial strains were purified and screened for their abilities to produce considerable quantities of hyaluronidase. The most potent strain was identified on the basis of the morphological, biochemical and molecular characteristics.

### 2.2. Cultural conditions

#### 2.2.1. Maintenance of the strains

The strains were maintained refrigerated at 4 °C on nutrient agar medium after incubation for 48 h at 37 °C. The strains were monthly subcultured.

### 2.3. Identification of the microorganisms

The utilized strain was identified on the morphological, the biochemical and the molecular basis. The strain was found to be belonged to *Staphylococcus* genus. The genomic DNA was isolated as described by Arciola et al. [11]. The 16s ITS region was amplified using the primer 5-AGAGTTTGATCCTGGCTGAG-3 [8] and 5-CAAGGCATCCACCATCCACCGT-3 [12]. The techniques performed as that described by Sudagidan et al. [13]. PCR was carried out using BioRad Thermo Cycler according to the amplification conditions of Sudagidan et al. [14]. Identification of the strain on species level was determined by sequencing of the genes. The ITS sequence identified the species of the strain [15]. An automated gene sequencer Perkin Elmer ABI prism7700 was used to sequence the gene. Database (Gene bank) was searched for the sequences similar to 16s rDNA and a phylogenetic tree was established using Blast NCBI using MEGA5.22 software.

### 2.4. Hyaluronidase production

Six different media were examined for their suitability for production of hyaluronidase:

Medium I (g/l): Glucose (5), $NH_4Cl$ (5), $MgSO_4$ (0.5), $KH_2PO_4$ (1.0), $CaCl_2$ (0.5) and peptic digest tissue (0.5) and whey (292 m/l). pH 5.8.
Medium II (g/l): Peptic digest of animal tissue (5), sod. chloride (5), beef extract (1.5), yeast extract (1.5), casein enzyme hydrolysate (1), $KH_2PO_4$, Magnesium sulfate (3), hyaluronic acid (0.01). pH 5.8
Medium III (g/l): Molasses, $(NH_4)_2SO_4$ (1) and $KH_2PO_4$ (0.5). pH 5.8
Medium IV (g/l): Beef extract 1.5, yeast extract 3.0, peptone 6.0, glucose 1.0
Medium V (g/l): Casamino acids 7.5, yeast extract 10.0, tri sodium citrate 3.0, KCl 2.0, $MgSO_4{\cdot}7H_2O$ 20.0, $FeCl_2$ 0.023
Medium VI: (g/l): Peptone 10.0, meat extract 2.4 $NaCl_2$.

Fractions of 50 ml volume from each medium were dispersed in 250 ml Erlenmeyer flask flasks then inoculated with a bacterial suspension (4%) and incubated on shaker incubator (Sanyo Gallenkamp) at 200 rpm shaking speed, 37 °C for 48 h or as specified.

**Table 1**
The levels of the variables for Plackett–Burman model.

| Variables | Variables levels | |
|---|---|---|
| | −1 | +1 |
| $X_1$: whey (ml/l) | 146 | 350 |
| $X_2$: peptic digest tissue (g/l) | 2.5 | 7.5 |
| $X_3$: glucose (g/l) | 2.5 | 7.5 |
| $X_4$: amm. chloride (g/l) | 2.5 | 7.5 |
| $X_5$: mag. sulphate (g/l) | 0.25 | 0.75 |
| $X_6$: pot. dihyd. phosphate (g/l) | 0.5 | 1.5 |
| $X_7$: calcium chloride (g/l) | 0.25 | 0.75 |
| $X_8$: pH | 6 | 7 |
| $X_9$: incubation periods (h) | 24 | 72 |
| $X_{10}$: inoculum size (%, v/v) | 2 | 6 |
| $X_{11}$: hyaluronic acid (g/l) | 0.005 | 0.015 |

Level of the different eleven variables ($X_1, X_2, X_3, \ldots$) including medium composition and culture conditions.

### 2.5. Protein determination

The protein content of the enzyme preparations was determined by the method of Lowry et al. [16].

### 2.6. Hyaluronidase assay

Hyaluronidase activity was measured by turbidity reduction [17]. Hyaluronic acid is measured by its ability to form turbidity with an acid albumin solution. Turbidity is a function of hyaluronic acid concentration and can hence be related to enzyme activity. One unit is based on the change in absorbency (turbidity) at 540 nm [18,19].

### 2.7. Optimization of the production process

#### 2.7.1. Screening of the variables

Eleven variables including the medium composition and some cultural conditions were screened for their effect on the amount the produced enzyme using Plackett–Burman model. Each variable was tested at two levels high (+1) and low (−1) as shown in Table 1. The model is based on first order polynomial model:

$$Y = \beta + \sum \beta_i X_i$$

where $Y$ is the enzyme activity (U/ml), $\beta$ is the model intercept, $\beta_i$ is the linear coefficient and $X_i$ is the levels of the variables [10].

#### 2.7.2. Box–Behnken model

Box–Behnken model was used to determine the optimum concentrations of the most significant three factors after Plackett–Burman. Each variable was tested on three levels (+1), (0) and (−1) as shown in Table 2. The model is based on quadratic equation as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{12} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where, $X_1$, $X_2$, $X_3$ are variables, $\beta_0$ is constant, $\beta_1$, $\beta_2$, $\beta_3$ are linear coefficients, $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ are quadratic coefficients [20].

**Table 2**
The level of the variables for Box–Behnken model.

| Variables | −1 | 0 | |
|---|---|---|---|
| $X_1$: whey (ml/l) | 250 | 350 | 450 |
| $X_2$: glucose (g/l) | 5 | 7.5 | 10 |
| $X_3$: inoculum size (%, v/v) | 5 | 6 | 7 |

The levels of the significant variables for the Box–Behnken model.

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