

Optimization of Auto-induction Conditions for the Heterologous Expression of a Maltogenic Amylase in *Escherichia coli*

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Abstract

Background and Objectives: Auto-induction is usually employed to achieve high cell density and overproduction of proteins with a simple and low-cost operation. The efficiency of heterologous protein expression in *Escherichia coli* is determined by different parameters. Interactions between these parameters usually complicate the identification of those that contribute more to the improvement of protein expression. As optimal implementation of the auto-induction considerably relies on both the composition of the auto-induction medium and induction conditions, the present study focused on the optimization of related culture parameters through response surface methodology.

Materials and Methods: In the first step, the optimum culture temperature and auto-induction duration were determined with the aim of achieving the highest specific activity. Then the culture composition was optimized through response surface methodology considering the concentration of carbon sources, glucose and lactose, as the variables for the simultaneous maximizing of the Maltogenic Amylase volumetric yield and specific activity.

Results and Conclusion: Expression of recombinant Maltogenic Amylase under optimum conditions in the shake-flask cultures of *Escherichia coli* harboring pET 28a increased by 1.7 folds in comparison with an un-optimized auto-induction culture. The Maltogenic Amylase specific activity and volumetric yield were found to be 34.93 U mg⁻¹ and 390.78 U ml⁻¹ at optimum conditions, respectively.

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1. Introduction

Amylases catalyze the hydrolysis of starch material playing a central role in carbohydrate metabolism. In comparison with other amylases that only hydrolyze α -D-(1,4)-glycosidic bonds, maltogenic amylases (MAases) (EC 3.2.1.133) display catalytic versatility [1]. It is shown that MAases readily hydrolyze cyclodextrins, pullulan, and starch. In addition to hydrolytic activity, substantial transglycosylation capacity was detected in MAases [2]. In 1984, Novozymes developed Maltogenase, a MAase as the first enzyme from a genetically modified organ-

ism for use in the starch industry [3].

Recently, MAases have been the center of an increasing amount of attention due to their applications in a variety of industrial and commercial processes, including baking industry, starch industries, synthesis of non-carcinogenic sweeteners, synthesis of novel carbohydrates, and development of new drugs for treatment of obesity, hyper-lipidemia, dental caries, and diabetes [4]. Some variants are especially useful in the production of ethanol from starch, and for textile resizing [5]. Recombinant MAase has been

expressed in different hosts [4], among which the Gram-negative bacterium *Escherichia coli* offers a means for rapid, high-yield, and economical production of recombinant proteins [6].

Among the limitations in the successful commercialization of recombinant proteins are the complications associated with the production and purification on a commercially viable level [7]. The efficiency of heterologous protein expression in *E. coli*, as an example, is determined by different parameters. Interactions between these parameters usually complicate the identification of those that contribute more to the improvement of protein expression. Some of these parameters are target protein specific (i.e. protein construct length and expression vector), while others are related to culture conditions (i.e. media type and cultivation time) or protein induction conditions (inducer concentration and induction time). Environmental parameters like oxygen-transfer rate, temperature, and pH affect both cell growth and protein expression [8].

Inducible expression systems in which T7 RNA polymerase transcribes coding sequences cloned under the control of a T7lac promoter efficiently produce a wide variety of proteins in *E. coli*. Investigation of factors that affect the stability, growth, and induction of T7 expression strains in shaking vessels led to the recognition of unintended induction of expression in complex media, called auto-induction [9]. Auto-induction relies on the diauxic response of *E. coli* when grown in multiple carbon sources such as glucose and lactose, resulting in the induction of lac promoter upon depletion of glucose [10]. This new method of induction was found to be superior to standard IPTG method. Auto-induction cultures are simply inoculated and grown to saturation, which is much more convenient than IPTG induction and particularly suitable for high throughput expression and solubility testing of multiple target proteins. Auto-induction proved to be usually applicable to the production of a wide range of proteins. The high cell densities achieved by auto-induction produce more target protein per culture volume than IPTG induction. Auto-induction is simple, effective and economical for heterologous protein production at nearly any scale [9].

According to the literature, the level of *LacI*, the oxygenation state of the culture, the medium composition, and the environmental factors like temperature have intense effects on the carbon consumption patterns necessary for successful implementation of the auto-induction process [9, 11,12].

Hence, it is crucial to determine the optimum culture temperature and auto-induction duration and study the interaction between the media components in determining the growth and enzyme production by the recombinant *E. coli* cells. The study of critical medium components through conventional one factor at a time procedure is time consuming, and does not allow identifying the effect of interactions between the

factors. To overcome this limitation, response surface methodology (RSM) has been commonly employed to study the effect of both primary factors and their mutual interactions. Central composite design (CCD) is a well-established, widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments. The objective of the present study was to improve the activity and yield of an *E. coli* expressed MAase by studying various medium components using CCD [13]. Despite the advantages associated with auto-induction in producing heterologous proteins, and the fact that MAase is an industrially and economically attractive enzyme, no report is available in the literature, as far as we know, about using auto-induction for MAase production.

2. Materials and Methods

2.1. Microorganism and media formulations

The microorganism used in the present work was a recombinant *E. coli* cell harboring MAase gene in pET28a expression vector. This microorganism was obtained from Dr. Sajedi's Lab (Biochemistry Department, Tarbiat Modares University). The cloning, expression, purification and characterization of the intra-cellular MAase from a thermophilic *Geobacillus* spp. isolated from Gheynarge hot spring (Soufian, East Azerbaijan, Iran) was done earlier [4]. The non-inducing and auto-induction media were derived from earlier reports on the development and use of auto-induction [9]. All media contained 50 µg ml⁻¹ of kanamycin.

2.2. Stock solutions

The stock solutions required for the auto-induction medium were originally defined by Studier [14]. All medium components were prepared using distilled water. The 20× NPS solution contained 66.0 g (NH₄)₂SO₄, 136.0 g KH₂PO₄, and 142.0 g Na₂HPO₄ per liter. This preparation was prepared heat-sterilized. The 50× 5052 solution was prepared from 250 g glycerol, 25 g D-glucose, and 100 g α-lactose in a final volume of 1 L. This preparation was prepared heat-sterilized. The 1000× kanamycin solution was made by dissolving 10 g of kanamycin in 50 ml of water for injection. The solution was filter-sterilized and stored at -20°C until use. A 40% glucose solution was prepared by adding 40 g of glucose to water to give a final volume of 100 ml and heat-sterilized [14].

2.3. Media for growth and expression

Media are conveniently assembled from sterile-concentrated stock solutions added to sterile ZY just before use. The name ZY will be reserved for 1 %wv⁻¹ N-Z-amine, 0.5 %wv⁻¹ yeast extract with no salt added. The ZYP-0.8G medium (50 ml) being used as the expression medium was assembled from the following sterile components: 46.5 mL ZY, 50 µL 1 M MgSO₄, 1 ml 40% glucose, and 2.5 ml 20× NPS. The ZYP-5052 medium (50 ml) being used as the

growth medium was assembled from the following sterile components: 46.5 ml ZY, 50 μ l 1M MgSO_4 , 1 ml 50 $\times$ 5052, and 2.5 ml 20 $\times$ NPS [9,14].

2.4. Cultivation

MAase BL21 cells harboring the MAase expression plasmid were grown at 37°C in ZYP-0.8G medium supplemented with 50 μ g ml^{-1} kanamycin. Pre-cultures were grown in sterile tubes in an incubator shaker, at 250 rpm for 8 hours. The ZYP-5052 medium was inoculated with 5×10^{-4} inoculations. In order to determine the optimum time and culture temperature, the incubation was carried out at three different temperatures (25, 30 and 37°C), and different times ranging from 8, 12, 16, 24 to 36 h. So, 15 experiments with defined culture times and temperatures were carried out. Optimum temperature and time were chosen by monitoring the MAase specific activity.

2.5. Statistical methods and experimental design

Two experimental factors, glucose and lactose, were chosen for the enhancement of MAase specific activity and volumetric yield by recombinant *E. coli*. The RSM methodology was used to study the effect of these two variables. The concentrations of glucose and lactose in the auto-induction medium were varied as part of a five-level, two-parameter factorial design in the following range of carbohydrate concentrations (wv^{-1}): glucose from 0 to 0.1%, and lactose from 0.1 to 0.9%. Glycerol concentration was maintained at 0.2% for all media formulations. Thirteen experiments, with four replications for the center point, were designed in order to study the effect of glucose and lactose concentrations. The MAase specific activity and volumetric yield after 12 h of incubation at 30°C were studied as responses, since there was no significant alteration in the MAase specific activity after this time. Data analysis was performed using Design Expert statistical software (ver. 6.0.9, Stat-Ease Inc, Minneapolis, MN). The responses were analyzed using the analysis of variance (ANOVA) combined with the Fischer's test to evaluate if a given term has a significant effect ($p \leq 0.05$). The optimum levels of the variables were obtained by graphical and numerical analysis using the Design Expert software [13].

2.5. Release and purification of recombinant MAase

The cells were harvested by centrifugation at 5000 $\times$ g for 15 min. The pellet was resuspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole, pH 8.0), disrupted by sonication in an ice bath (Dr. Hielscher UP400s, for 5 min at 50% amplitude and 0.2 cycle), clarified by centrifugation (14000 $\times$ g, 25 min, 4°C), applied on SDS-PAGE and, or stored at -70°C. Purification of His-tagged fusion proteins was carried out using a nickel-nitrile triacetic acid (Ni-NTA) agarose column. Bound proteins were eluted with buffer containing 50 mM NaH_2PO_4 ,

300 mM NaCl and 250 mM imidazole, pH 8.0. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% wv^{-1} polyacrylamide gel by the method of Laemmli. The protein concentration was determined according to the Bradford method [4].

2.6. MAase assay

MAase activity was assayed using the dinitrosalicylic acid method described by Miller. A mixture of 40 μ l of sodium-acetate buffer (50 mM, pH 6.0) and 50 μ l of 1% β -CD in the same buffer was pre-warmed at 60°C. Ten micro-liter of the purified enzyme solution with appropriate dilution was added and incubated at 60°C. The reaction was then stopped and colorized by adding dinitrosalicylic acid solution after 5 min. The mixture was boiled for 5 min in a water bath, cooled down to room temperature. Then the absorbance was measured at 540 nm. All experiments were performed in the aforementioned conditions [4]. One unit of α -amylase is defined as the amount of enzyme that liberates 1 μ mol of reducing groups per min.

3. Results and Discussion

3.1. Time courses and temperature optimizations

An important step for high-level protein production using high-cell-density bacterial expression culture is to optimize the expression conditions such as culture temperature and induction time both for IPTG-induction and auto-induction. This step is critical for the first-time expression of a new protein using high-cell-density expression method. First, the time courses of MAase specific activity at different temperatures (25, 30, and 37°C) were monitored [15]. Table 1 clearly demonstrates the importance of the induction time, indicating that MAase activity is increasing until 12 h, and the expressed MAase starts to degrade after 16 h. Furthermore, during the cultivation, the pH decreases because of acetate accumulation. The pH reduction may lead to significant instability of the plasmid, resulting in plasmid loss and significant reduction of the protein yield. In addition, for bacterial expression of a kanamycin-resistant plasmid, further reduction in pH may also result in degradation of kanamycin in the medium. This could result in the loss of kanamycin-resistant plasmids in the bacterial cells, thus the cell density can further increase without protein production or a very low protein yield. Besides, proteolytic degradation of the heterologous protein by the host cell proteases is much more probable at prolonged times [15]. Diauxic growth in a glucose-lactose medium affects the trends of specific activity alternation during time. After the depletion of glucose, the cAMP level rises sharply, and decreases rapidly when the cells enter the second exponential phase [16].

Table 1. Effect of the time courses and culture temperature of auto-induction on MAase specific activity

Temperature (°C)	MAase specific activity (U mg ⁻¹)				
	8 h	12 h	16 h	24 h	36 h
25	18.19	29.99	27.91	19.93	27.49
30	23.13	44.5	35.73	14.03	24.79
37	41.04	30.99	27.29	31.67	22.00

Table 2. Factors and their levels for the CCD

Factor	Low axial ($-\alpha = -2$)	Low factorial (-1)	Center (0)	High factorial ($+1$)	High axial ($+\alpha = +2$)
Glucose (%wv ⁻¹)	0	0.01	0.05	0.09	0.1
Lactose (%wv ⁻¹)	0.1	0.22	0.5	0.78	0.9

Table 3. CCD and the results obtained

Run	A: Lactose (%wv ⁻¹)	B: Glucose (%wv ⁻¹)	MAase Specific Activity (U mg ⁻¹)	MAase Volumetric Yield (U ml ⁻¹)
1	0.5	0.05	31	370
2	0.9	0.05	18.33	133
3	0.5	0.05	29.26	366.26
4	0.5	0.05	31.18	326.05
5	0.5	0.05	30.99	387.45
6	0.22	0.01	8.00	70.00
7	0.10	0.05	13.52	103.00
8	0.50	0.10	24.36	291.30
9	0.22	0.09	31.70	353.36
10	0.50	0.00	29.35	390.95
11	0.78	0.09	7.36	72.55
12	0.78	0.01	31.36	395.76

Table 4. ANOVA test for the quadratic models

		Sum of squares	DOF	Mean square	F-Value	p-Value	Lack of fit
MAase activity	Model	971.86	5	194.37	52.78	<0.0001	0.0603
	Residuals	22.09	6	3.68			
MAase yield	Model	1.963E+005	5	39254.75	69.53	<0.0001	0.6224
	Residuals	3387.64	6	564.61			

Table 5. Optimized process conditions and their results

Case	Target	Lactose (%wv ⁻¹)	Glucose (%wv ⁻¹)	MAase specific activity (U mg ⁻¹)	MAase volumetric yield (U ml ⁻¹)
MAase specific activity/Volumetric yield	Maximize	0.62	0.02	33.10	413.91

Table 6. Point prediction of the responses at the optimal conditions

Response	Prediction	SE mean	95% C.I. low	95% C.I. high	SE pred.	95% P.I. low	95% P.I. high
MAase activity	33.10	1.02	30.60	35.60	2.17	27.78	38.42
MAase yield	413.91	12.64	382.98	444.85	26.92	348.05	479.77

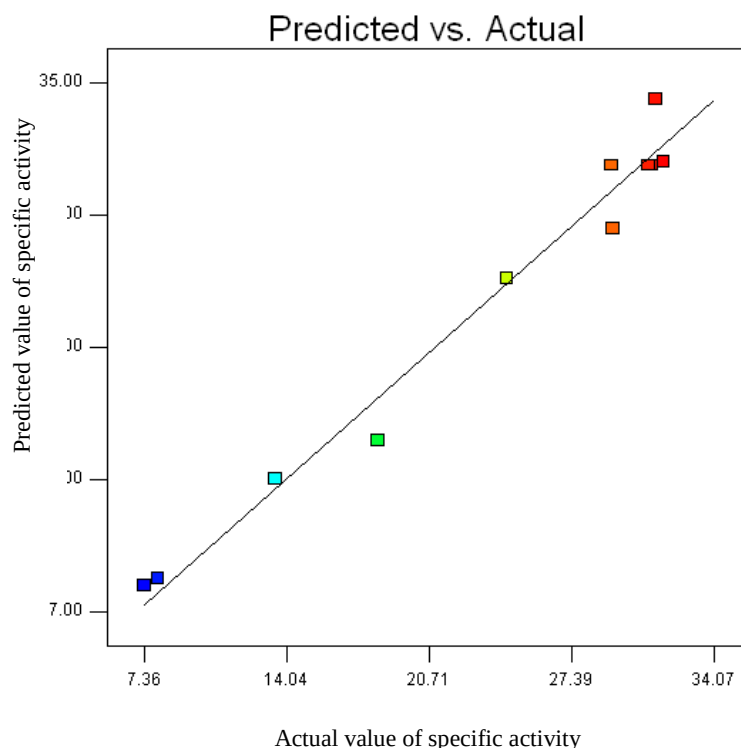


Figure 1: Predicted vs. actual values of specific activity.

3.2. Optimization of medium compositions by central composite design

3.2.1. Data analysis

The data obtained from the shake flask experiments were statistically analyzed to identify the significant main effects as well as the interaction effects. ANOVA of the experimental results was carried out to decide upon the significant and insignificant effects and thus obtain the best possible regression model [17].

3.2.2. Design

A CCD with five coded levels for the two factors, lactose (A) and glucose (B), was used for this purpose. The levels of variables for the CCD were based on the preliminary results. The range of the factors is given in Table 2. The experimental designs and the results obtained for the enzyme specific activity and volumetric yield are presented in Table 3 [13].

3.2.3. Fitting models

Eqs. (1) and (2) were obtained from the results of 13 shaking flask runs using the Design-Expert 7.1.4 software. It was found that a quadratic model could best fit the experimental data; therefore, coefficients with p-values greater than 0.05 were eliminated:

Eq. 1:

$$\text{Specific activity} = +31.49 - 11.93 AB - 8.23 A^2 - 2.76 B^2$$

Eq. 2:

$$\text{Volumetric yield} = +359.36 - 22.6 B - 151.64 AB - 122.34 A^2$$

The ANOVA results for the quadratic models are shown in Table 4. The F-value for the models shows that the models are significant at less than a 0.05% level (i.e., at 95% confidence interval).

Eq. (1) shows that negative quadratic effect of the lactose and glucose concentrations with negative effect of interaction between the variables, exerting an important effect on the specific activity of MAase in the auto-induction method. It is obvious from Eq. (1) that the negative quadratic effects and the negative interactions between parameters should also be considered. In Eqs. (1) and (2), the lactose concentration (A) does not have an important effect on the MAase specific activity and volumetric yield.

3.2.4. Determination of model adequacy

It is to be noted that polynomial models are reasonable approximations of the true functional relationship over relatively small regions of the entire space of independent variables [16]. Fig. 1 represents predicted vs. actual values of MAase specific activity. The clustering of the points around the diagonal line indicates a satisfactory correlation between the experimental data and the predicted values, confirming the robustness of the model (data for MAase volumetric yield is not shown).

3.2.5. Contour plots and response surfaces

Figures 2–3 show the three-dimensional response surfaces of responses as well as the contour plots of the relationship between different parameters at the optimized values. According to the models, interaction between variables has significant effect on the responses; therefore, the results were presented and discussed in terms of interactions. It is indicated that MAase activity depends more on the interaction between lactose and glucose concentrations and the quadratic effect of lactose concentration rather than on other parameters. It is evident from Figs. 2 and 3 that, in the constant lactose concentration ($0.05\% \text{wv}^{-1}$), the glucose concentration has a negative effect on the MAase volumetric yield. Because increasing the gluc-

ose concentration is accompanied with increasing the cell density. So, lactose and glycerol concentrations should be increased simultaneously. But these carbon sources' concentrations are constant. Accordingly, at a constant lactose and glycerol concentration, increasing the glucose concentrations was accompanied with decreasing the volumetric yield of MAase in agreement with Li results [18].

In the case for the volumetric yield of MAase, interaction between the glucose and lactose concentrations has negative effect on this parameter. At the absence of glucose or low glucose concentration, lactose is used as an energy source, so higher lactose concentration is desirable.

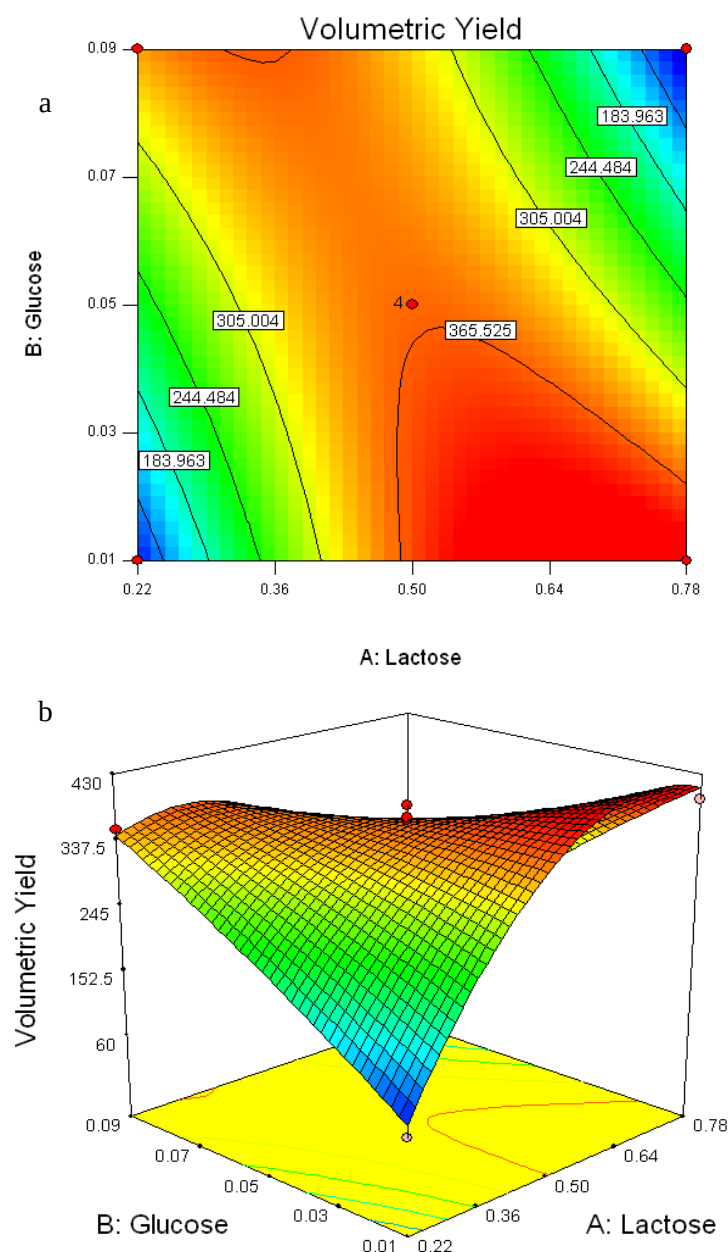


Figure 2. (a) Contour plots, and (b) response surface for MAase volumetric yield.

On the other hand, at high glucose concentrations, the pH reduction may lead to significant instability of

the plasmid, resulting in plasmid loss, so lower lactose concentration is needed.

3.2.6. Determination of optimum conditions

The optimized conditions of the auto-induction process obtained from the shake flask experiments (Table 5) were %wv⁻¹ of glucose concentration and %wv⁻¹ of lactose concentration, which gives a maximum MAase specific activity of U mg⁻¹ and MAase volumetric yield of U ml⁻¹. The aim of optimization was to maximize the MAase specific

activity and volumetric yield simultaneously within the ranges of variables.

The desirability of the process optimization was found to be 0.904. It is worth noting that the goal of optimization is to find a good set of conditions that will meet all of the goals, not just get to a desirability value of 1.0. Desirability is simply a mathematical method to find the optimum prediction of the responses at the optimal settings. The final step of the

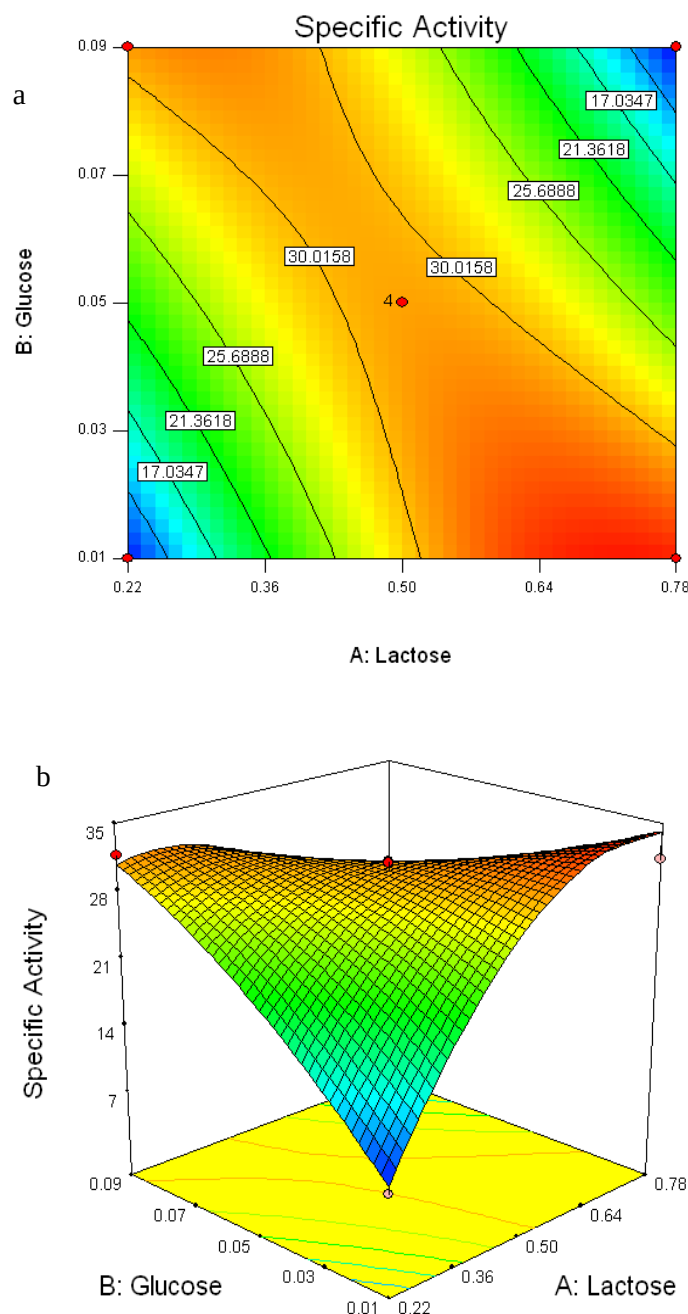


Figure 3. (a) Contour plots, and (b) response surface for MAase specific activity.

experiment before doing the confirmation runs to verify that the prediction is the point prediction (Table 6), which allows entering the levels for each factor or component into the current model. The software then calculates the expected responses and associated

confidence intervals based on the prediction equation shown in the ANOVA output. The 95% confidence interval (C.I.) is the range in which the process average was expected to fall 95% of the time, while the 95% prediction interval (P.I.) is the range in which

it was expected that any individual value to fall 95% of the time. As shown in Table 6, the P.I. is larger (a wider spread) than the C.I. since more scatter in the individual values is expected than in the averages. In this table, SE mean refers to the standard deviation associated with the prediction of an average value at the selected component levels, while the standard deviation associated with SE prediction indicates the prediction of an individual observation at the selected factor or component levels.

3.2.7. Confirmatory experiments

To test the validity of the optimized conditions given by the model, an experiment was carried out with the parameters suggested by the model. The results indicated that the experimental values are very close to the predicted values; hence, the model is successful in predicting the responses. The MAase specific activity and volumetric yield were found to be 34.93 U mg^{-1} and 390.78 U ml^{-1} , respectively; therefore, the formulated model is valid and accepted.

4. Conclusion

In this study, the auto-induction approach was employed and optimized for production of MAase in *E. coli*. The effect of temperature, time, and glucose and lactose concentrations was investigated. First, the best culture temperature and auto-induction duration for attaining maximum MAase specific activity were determined. After carrying out time courses at different temperatures, RSM was applied for finding the optimal concentrations of lactose and glucose. The results revealed that culture with 30°C and 12 h cultivation results in the highest MAase specific activity. The RSM results showed that the negative effect of interaction between variables exerts an important effect on both the enzyme yield and specific activity. Maximum amounts of yield and specific activity for MAase were obtained as 413.91 U ml^{-1} of culture and 33.10 U mg^{-1} of protein, respectively, using glucose and lactose with 0.02% and 0.62 %wv⁻¹ concentrations.

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6. Conflict of interest

The authors declare no conflict of interest.

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