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Original paper

Recombinant Glutamate Decarboxylase to Increase Gamma-aminobutyric Acid Production

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Abstract

Due to the increasing global demand for Gamma-aminobutyric acid (GABA) in the food and pharmaceutical industries, the expression of the recombinant Glutamate decarboxylase (GAD) and its industrial production is currently requested. Culture conditions were optimized to increase the expression level of the recombinant enzyme in different pH, temperature, incubation time, aeration levels, inoculation concentrations, concentrations of IPTG, and several carbon sources using the RSM based on a central composite design. According to the results of the quadratic regression equation, recombinant *Escherichia coli* BL21 (DE3) had the highest GAD expression at pH=7.2, aeration at about 120 rpm, inoculation concentrations of about 3% v/v, 2.25 mM IPTG, 37 °C, and 6 h of incubation time in the presence of 0.2% glucose. Using pure glucose as a carbon source on an industrial scale is not cost-effective for producing recombinant proteins. Therefore, using low-cost carbon sources such as corn syrup and molasses with concentrations of 1.5 and 5.65% (w/v) is an efficient method for the industrial production of recombinant GAD. The concentration of purified recombinant GAD in carbon sources of 0.2% glucose, 1.5 corn syrup and 5.65% molasses was 2.155, 2.07 and 1.96 mg/mL, respectively. In this way, the global need for GABA can be met by the industrial production of GAD.

Keywords
Gamma-aminobutyric acid (GABA)
Glutamate decarboxylase (GAD)
Industrial production
Optimization

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Introduction

Gamma-aminobutyric acid (GABA) is one of the important amino acids in treating nervous system issues such as insomnia, stress, and depression and restoring damaged nervous tissue. Glutamate decarboxylase (GAD) catalyzes GABA biosynthesis reaction. It is an intracellular pyridoxal phosphate-dependent enzyme that decarboxylates glutamate and produces GABA (Wu, 2017). The discovery of the relationship between some neurological disorders and physiological abnormalities and the amount of GABA in people's bodies, as well as the relationship between receiving GABA and healing these disorders, has increased the demand for using GABA. Therefore, it has received much attention in the pharmaceutical and food industries (Yarabbi, 2020). The use of recombinant enzymes compared to the whole cell to produce GABA-rich foods on an industrial scale has the following advantages:

A) The specificity of enzyme reactions that prevent the production of unwanted by-products.

B) Enzymes are degradable.

C) Enzymes can activate at neutral pH and high temperatures that cause energy storage (Chen, 2012; Yarabbi, 2021).

The growth of recombinant *Escherichia coli* BL21(DE3) and the expression level of recombinant GAD enzyme gene are strongly influenced by physical parameters such as pH of the culture medium, temperature, time of incubation, aeration level, inoculation rate, and concentrations of Isopropyl β- d-1-thiogalactopyranoside (IPTG), as well as the composition of the culture medium, especially the carbon source (Shi, 2014). Considering the industry demand and the high production cost of commercial enzymes, the development of new processes to increase the efficiency of enzyme production and reduce production costs has been considered (Chen, 2012; Roshanak, 2020). About 30-40% of the cost of enzyme production is related to the culture medium. The carbon source is the most critical component of the bacterial culture medium. Therefore, it directly affects the growth and activity

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of bacteria (Tyagi, 2016; Yarabbi, 2021). For the expression of the recombinant GAD enzyme by *E. coli* BL21 bacteria on a laboratory scale, a Luria Bertani (LB) culture medium (containing 5 g/L yeast extract, 10 g/L tryptone, and 5 g/L sodium chloride) along withglucose is used (Sezonov, 2007). Pure glucose is rarely used in industrial applications, mainly due to its high cost. For industrial production of recombinant glutamate decarboxylase (GAD), this medium is expensive. Therefore, cheap carbon sources such as molasses and corn syrup were used to reduce the cost of recombinant enzyme production, improve its production efficiency and naturally increase the production efficiency of GABA. Therefore, optimizing the cultivation conditions of recombinant *E. coli* BL21(DE3) is essential for production of recombinant GAD on an industrial scale.

Materials and methods

Expression vector pET-21a (+) (IBRC V10204E), *E. coli* BL21 (DE3) (IBRC-M 11187) were provided from Iranian Biological Resource Center (IBRC, Tehran, Iran). Bradford reagent for enzyme quantification, 1 kb DNA Ladder, 245 kDa protein Ladder, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A pre-equilibrated Ni-NTA affinity column was from Bio-Rad (CA, USA).

Microbial strain and investigation of optimal expression conditions of recombinant GAD

Recombinant *E. coli* BL21(DE3) cells containing pET21a (+)-GAD vector obtained from our previous study (Yarabbi, 2021). They were grown on LB broth medium (0.5% yeast extract, 1% peptone, 1% NaCl) with 100 µg/mL ampicillin. Protein expression was induced by adding IPTG into the culture when the optical density of the culture at 600 nm reached 0.6-0.65 (Chen, 2012; Roshanak, 2023). After the growth of the recombinant *E. coli* BL21 cells, they were harvested and their absorbance was evaluated at 600 nm (Chen, 2012). To optimize the culture medium conditions for the growth and activity of *E. coli* BL21 parameters such as culture medium pH (6.5-7.9), incubation time (3 to 9 h), incubation temperature (30-44 ˚C) was evaluated based on the treatments determined by Design Expert software version 11.0 of the central composite design. Moreover, the effect of the aeration level during fermentation on the expression of the recombinant GAD was studied by incubating the culture in shaking incubators, at various shaking speeds ranging from 0 to 200 rpm. 1 % (v/v) of the activated culture was inoculated into an LB broth medium. Samples were harvested and *E. coli* growth and recombinant GAD expression were investigated (Salehi Jozani, 2015). Isopropyl β- d-1-thiogalactopyranoside (IPTG) is a molecular biology reagent used to induce the expression of recombinant proteins where the gene is under the control of the lac operator. The best time to add the inducer is the exponential phase (Tolia, 2006). Low concentrations of IPTG induce protein expression at a slower speed but allow more protein to be folded correctly. If the induction time become longer, the expression level of recombinant protein will increase. Although, it may cause heterogeneity of the target protein, proteolysis, and low protein yield (Einsfeldt, 2011; Sørensen, 2005). Therefore, different concentrations of IPTG should be tested to determine the optimal concentration of IPTG for recombinant GAD expression.

Effect of different carbon sources on recombinant GAD expression

The effect of the type of carbon source on the expression level of recombinant GAD was investigated. Corn syrup is a clear liquid containing a wide range of carbohydrates produced from corn starch during acid or enzymatic hydrolysis. In the acid hydrolysis process, corn syrup is created by combining corn starch with dilute hydrochloric acid and then heating it (Mousavi Nasab, 2010). In the enzymatic hydrolysis process, an alpha-amylase enzyme is first added to a mixture of cornstarch and water. This enzyme breaks down the starch into oligosaccharides and then breaks it into glucose molecules by adding glucoamylase enzyme (Samadlouie, 2019). Molasses contains 70-77% dry matter, of which 50% is sugar. The predominant sugar in molasses is sucrose (46%). The amount of crude protein in molasses is 4-7% and its major part includes non-protein nitrogenous compounds such as amines and betaine (Gomaa, 2014). Most microorganisms can hydrolyze sucrose into two monosaccharides, glucose and fructose, and use it as a carbon source in their metabolic pathways. Since molasses is an industrial effluent from sugar factories, it is one of the cheapest, most accessible, and most nutritious substrates for microorganisms (Mousavi Nasab, 2010). Therefore, LB broth culture medium containing different concentrations of glucose (0.1, 0.2, 0.3, and 0.4% w/v), corn syrup (1.75, 3.5, 5.25, and 7%w/v) and sugar beet molasses (0.75, 1.5, 2.25, and 3% w/v) were prepared. This was based on the response surface method of the univariate design. All samples were examined in terms of the level of expression of the GAD gene by measuring the absorbance at a wavelength of 600 nm. Tricalcium phosphate ($Ca_3(PO_4)_2$) was used to treat molasses. pH of molasses was adjusted to 7 by adding 0.1 normal sodium hydroxide (NaOH) and then treated with 2% (w/v) tricalcium phosphate. Then it was placed in an autoclave at 120 ˚C for 5 min. The obtained solution was centrifuged at a speed of 3000 rpm at a temperature of 25 ˚C for 15 min. The supernatant obtained from the centrifuge was used as a carbon source (Ramezani, 2011).

GAD production and protein content assay

The cell pellet from the final treatment in optimal condition was washed with double-distilled water and then resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF, pH: 8.0) and followed by 6 cycles of intermittent sonication at 25 W for 30 s. Cell debris was removed by centrifugation at 12000 g for 20 min at 4 ˚C and the supernatant containing the recombinant GAD was collected (Einsfeldt, 2011; Roshanak, 2021). The sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% separating and 5% stacking. The SDS-PAGE method confirmed the GAD enzyme expression in the optimal culture medium for *E. coli* BL21 (Chen, 2012; Yarabbi, 2021). The recombinant protein was purified by Ni2+-NTA

affinity chromatography (Einsfeldt, 2011). The recombinant GAD concentration was determined using the Bradford method (Bradford, 1976).

Enzyme activity determination

To evaluate the activity of the recombinant GAD enzyme, the method of Li (2009) was used. The supernatant containing recombinant GAD was added to a mixture of 20 mM monosodium glutamate, 5-pyridoxal phosphate (PLP (0.1 mM), and 2 mM phosphate/citrate buffer. Then it was placed in a shaker incubator at 45 °C for 1 h. 2 µL of the sample was placed at the bottom of the thin layer chromatography (TLC) plate. The TLC plate was placed in a solution consisting of ninhydrin with butanol, acetic acid, and distilled water at a ratio of 5:3:2 for 90 min. It was dried at 70 °C for 80 min. TLC technique was performed for standard GABA (1000 mg/L) and monosodium glutamate (20 Mm) as control samples (Li, 2009).

Results and discussion

After determining the low and high levels of independent variables pH, temperature, incubation time, aeration, inoculation concentration, and IPTG concentration in the response surface method of Design-Expert software (Table 1) and choosing the central composite design, the most appropriate fitted model was determined. Then the linear and quadratic effects of each variable as well as their mutual effect on the recombinant GAD enzyme expression were evaluated. Since the quadratic fitted model had $R^2 = 0.88$ and $R^2_{adj} = 0.85$, its BIC and AIC values were lower than other models (linear, 2Fl, cubic and quartic), this demonstrated that there was a high correlation between the results of the experimental and the predicted by the Design Expert software (Fig. 1). Therefore, the proposed quadratic polynomial model is suitable for examining the changes in the measured OD_{600nm} and predicting the expression level of the recombinant GAD (Table 2). The quadratic polynomial equation resulting from the regression analysis is as follows. In this Eq. (1), OD_{600nm} is a function of the value of the independent variables:

(1)

$OD_{600nm} = -38.36 + 0.3\ Time + 0.3\ Temperature + 0.002\ Aeration + 0.27\ Inoculation\ concentration + 0.25\ IPTG\ concentration - 0.64\ pH^2 - 0.012\ Time^2 - 0.004\ Temperature^2 - 0.03\ Inoculation\ concentration^2 - 0.085\ IPTG\ concentration^2$

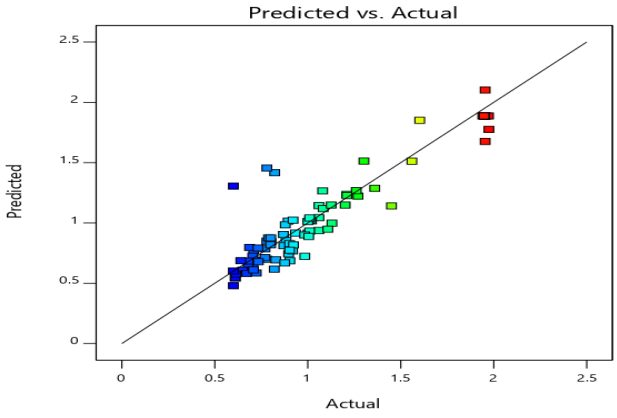


Fig. 1. Experimental residual values according to predicted values.

Table 1. Factors evaluated for expression of recombinant GAD by *E. coli* BL21.

Name	Units	Type	Low	High
pH		Factor	6.50	7.90
Time	h	Factor	3.00	9.00
Temperature	°C	Factor	30.00	44.00
Aeration	rpm	Factor	0.00	200.00
Inoculation concentration	%	Factor	1.00	5.00
IPTG concentration	%	Factor	0.50	4.00
Absorbance OD_{600nm}	Au	Response	0.60	1.97

Table 2. Variance analysis of the results of recombinant GAD expression by *E. coli* BL21

Source	Term df	Error df	F-value	p-value	
Whole-plot	2	6.016	20.18	0.00214	significant
a-pH	1	5.022	2.065	0.20994	
a^2	1	7.4395	38.37	0.00035	
Subplot	25	45.905	5.725	0.00008	significant
B-Time	1	55.89752	30.54682	0.000086	
C-Temperature	1	55.89752	14.46989	0.00035	
D-Aeration	1	55.89752	14.95021	0.00029	
E-Inoculation concentration	1	55.89752	11.50786	0.00127	
F-IPTG concentration	1	55.89752	6.415849	0.0141	
aB	1	55.89752	1.298473	0.259	
aC	1	55.89752	0.17077	0.681	
aD	1	55.89752	0.735676	0.394	
aE	1	55.89752	0.002659	0.9590	
aF	1	55.89752	1.089182	0.30114	
BC	1	55.89752	0.224912	0.63716	
BD	1	55.89752	0.101652	0.75104	
BE	1	55.89752	0.053675	0.81763	
BF	1	55.89752	2.672625	0.1077	
CD	1	55.89752	0.180509	0.67256	
CE	1	55.89752	1.358742	0.2487	
CF	1	55.89752	0.120012	0.7303	
DE	1	55.89752	0.294326	0.5896	
DF	1	55.89752	0.748188	0.39074	
EF	1	55.89752	0.004368	0.9475	
B^2	1	44.1322	5.640898	0.0219	
C^2	1	44.1322	16.54319	0.00019	
D^2	1	44.1322	0.535142	0.46832	
E^2	1	44.1322	6.908959	0.0117	
F^2	1	44.1322	29.42435	0.000002	
-2 Log Likelihood	9.551463	BIC	49.74464		
AIC	27.55146	AICc	29.88913		
R^2	0.88	Adjusted R^2	0.85		
Std. Dev.	0.210108	Mean	1.054092		
C.V.%	19.93262				

Effect of pH on recombinant GAD expression

Using the predicted statistical model, it is possible to estimate OD_{600nm} at different pH values, temperature, incubation time, aeration, inoculation concentration, and IPTG concentration variables. The pH of the culture medium had a greater effect on the expression of recombinant GAD because its linear coefficient was higher than other variables. The pH of the culture medium can affect cell morphology, cell membrane function, nutrient absorption, and product biosynthesis. To investigate the effect of pH on the expression level of recombinant GAD and determine the optimal pH, *E. coli* BL21 bacteria containing PET 21 a (+)-GAD plasmid in LB broth culture medium with pH of 6, 6.5, 7.2, and 7.9 and 8 determined by the response surface method. The maximum expression of the recombinant GAD was observed at pH = 7.2, and at this point, the light absorbance at 600 nm was 1.947. The activity of recombinant *E. coli* BL21 decreased

significantly between pH < 6.8 and pH < 7.7 (Fig. 2A). The decrease in bacterial activity at pH less than 6.8 could be explained by the fact that bacteria break down sugars during growth and activity and produce a mixture of acids such as lactate, succinate, ethanol, acetate, and carbon dioxide. A sharp decrease in the pH of the environment limits the growth of bacteria. In general, by changing the pH of the culture medium, the concentration of hydrogen ions in the culture medium of bacteria changes and thus affects its growth and activity. According to the obtained results, pH = 7.2 was recognized as the optimal pH for the growth and activity of *E. coli* BL21 (Fig. 2A).

Effect of inoculation concentration on recombinant GAD expression

The inoculation concentration affected *E. coli* BL21 growth and recombinant GAD expression. Good results were observed at inoculation concentrations of about 3% v/v. These results were quite comparable with those reported for *Bacillus thuringiensis* (Salehi Jozani, 2015), alkaline protease production from *Bacillus* sp. RGR-14 (Abdella, 2023; Puri, 2002) and production of acidic lipase by *Aspergillus* (Mahadik, 2002; Wei, 2023), that bacterium growth and protein production were at an inoculation about 1 to 4% (v/v). A low percentage of inoculation leads to a low number of bacterial cells in the culture medium; while the excessive increase in the percentage of inoculation also causes a decrease in the activity of bacteria due to the limitation of nutrient resources (Abdella, 2023). As shown in Figs. (2E), (3D), (4C), and (5F), increasing the inoculation percentage from 3 to 3.5% did not have a significant effect on the growth of *E. coli* BL21 and the expression of recombinant GAD ($P \geq 0.05$). Therefore, according to economic efficiency, the amount of 3% inoculation was determined as the optimal percentage of inoculation for *E. coli* BL21.

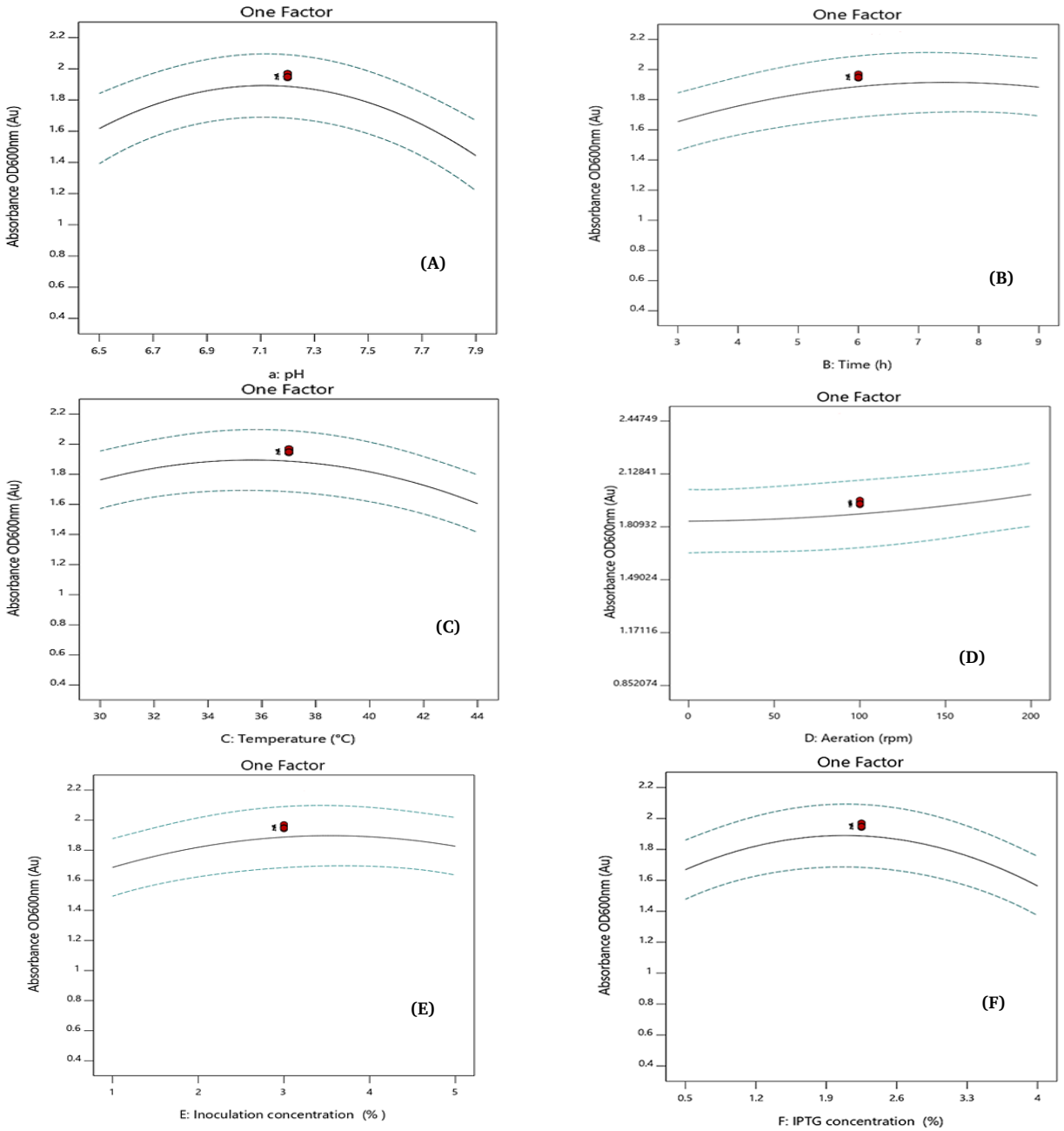


Fig. 2. Effect (A) Temperature of incubation, (B) Time of incubation, (C) pH of the medium, (D) Aeration, (E) Inoculation concentration, and (F) IPTG concentration on the expression level of recombinant GAD.

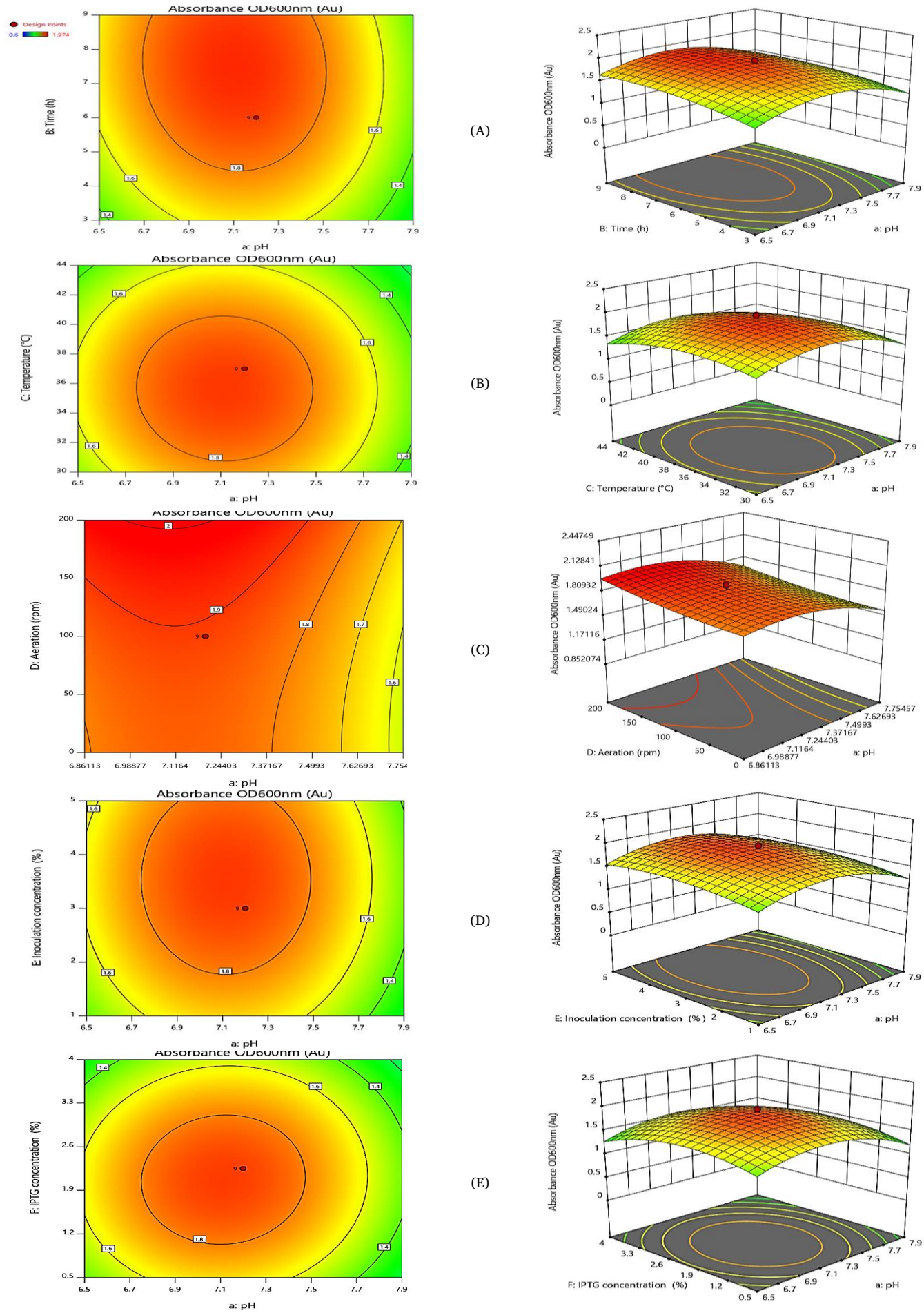


Fig. 3. Contour and three-dimensional response surface diagrams of changes in (A) pH and incubation time, (B) pH and incubation temperature, (C) pH and aeration, (D) pH and inoculation concentration, (E) pH and IPTG concentration on the expression level of recombinant GAD.

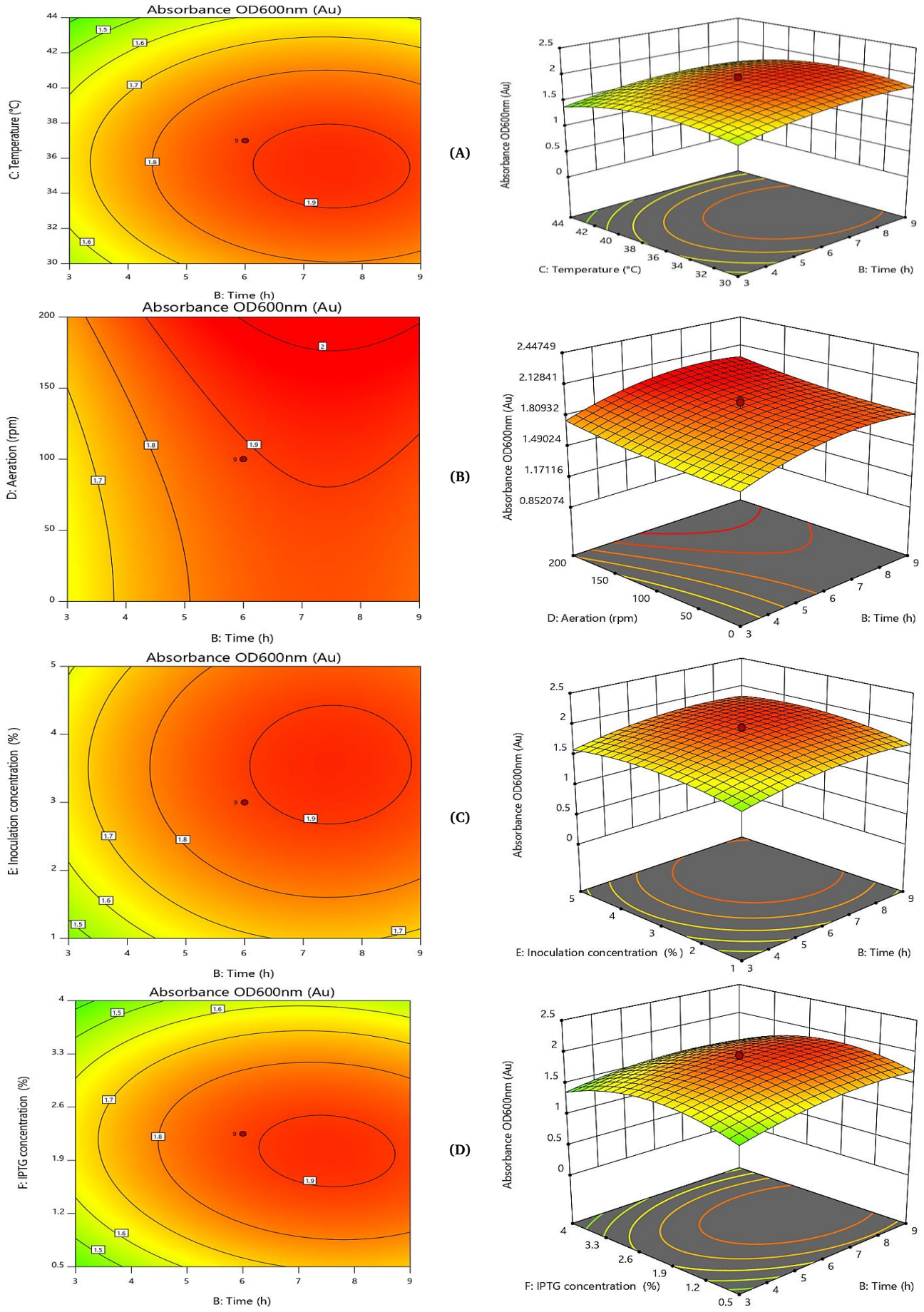
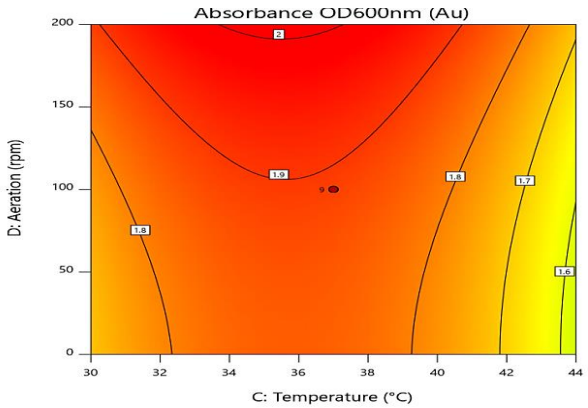
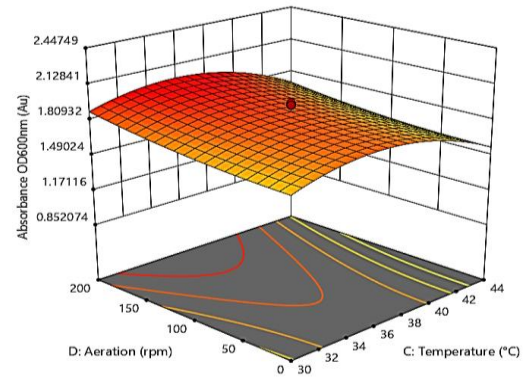
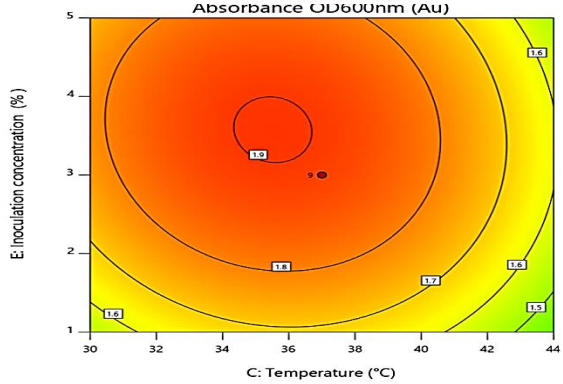


Fig. 4. Contour and three-dimensional response surface diagrams of changes in (A) Incubation time and temperature, (B) Incubation time and aeration, (C) Incubation time and inoculation concentration, (D) Incubation time and IPTG concentration on the expression level of recombinant GAD.

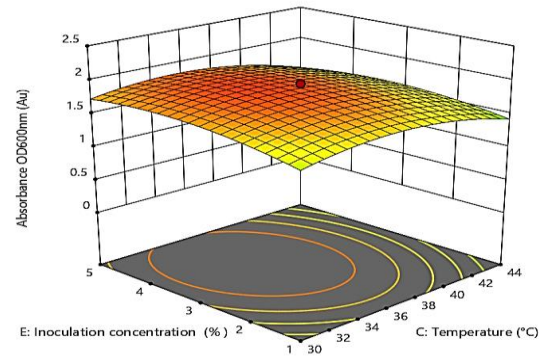

Absorbance OD600nm (Au)
D: Aeration (rpm)
C: Temperature (°C)

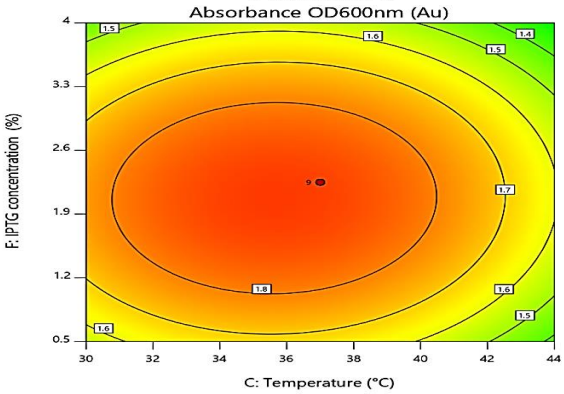
(A)


Absorbance OD600nm (Au)
D: Aeration (rpm)
C: Temperature (°C)

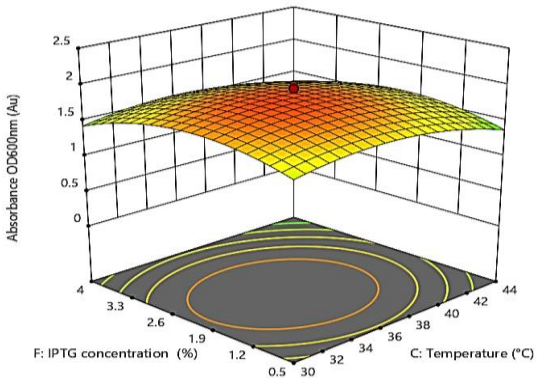

Absorbance OD600nm (Au)
E: Inoculation concentration (%)
C: Temperature (°C)

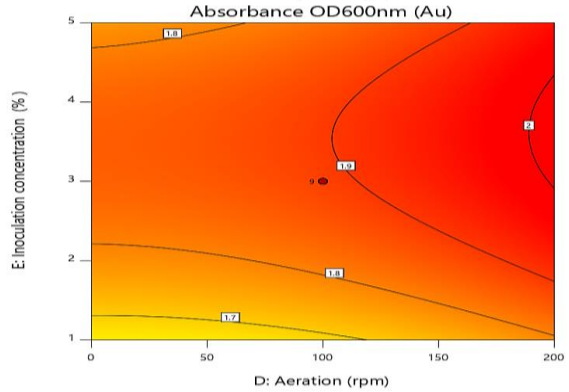
(B)


Absorbance OD600nm (Au)
E: Inoculation concentration (%)
C: Temperature (°C)

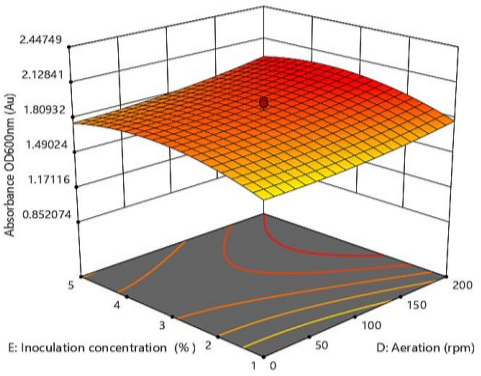

Absorbance OD600nm (Au)
F: IPTG concentration (%)
C: Temperature (°C)

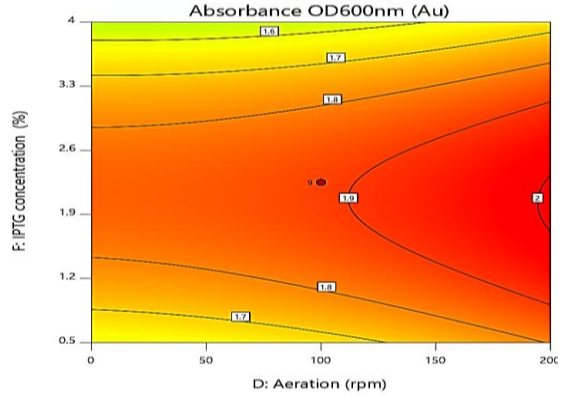
(C)


Absorbance OD600nm (Au)
F: IPTG concentration (%)
C: Temperature (°C)

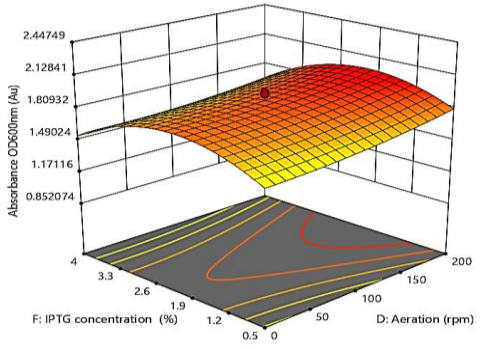

Absorbance OD600nm (Au)
E: Inoculation concentration (%)
D: Aeration (rpm)

(D)


Absorbance OD600nm (Au)
E: Inoculation concentration (%)
D: Aeration (rpm)


Absorbance OD600nm (Au)
F: IPTG concentration (%)
D: Aeration (rpm)

(E)


Absorbance OD600nm (Au)
F: IPTG concentration (%)
D: Aeration (rpm)

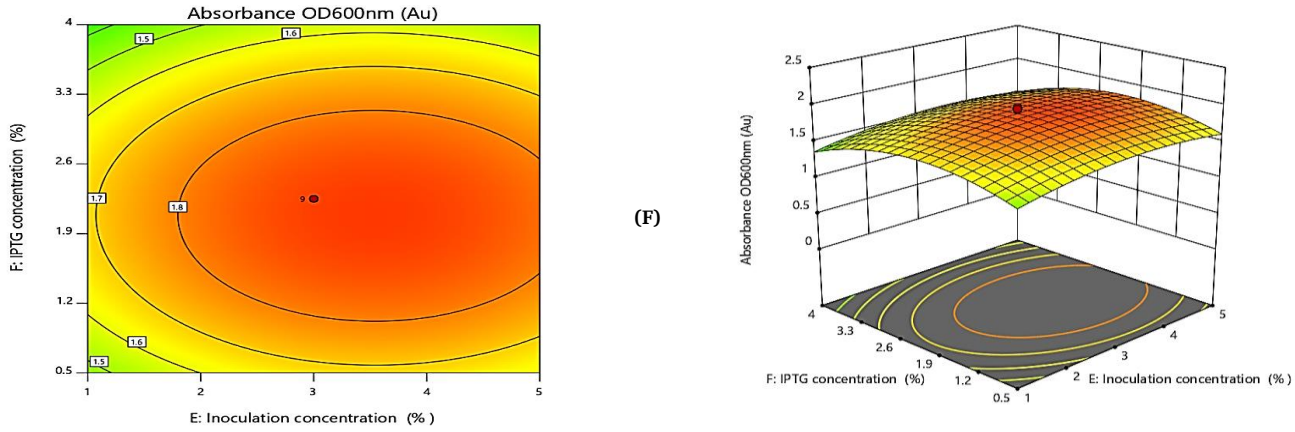


Fig. 5. Contour and three-dimensional response surface diagrams of changes in (A) Incubation temperature and aeration, (B) Incubation temperature and inoculation concentration, (C) Incubation temperature and IPTG concentration, (D) Aeration and inoculation concentration, (E) Aeration and IPTG concentration, and (F) Inoculation concentration, and IPTG concentration on the expression level of recombinant GAD.

Effect of IPTG concentration on recombinant GAD expression

As can be seen in Figs. (2F), (3E), (4D), and (5C,E,F), concentrations lower than 1.8 mM of IPTG reduced the recombinant GAD expression. A concentration higher than 3 mM of IPTG showed adverse effects on cell growth of *E. coli* BL21 and vector expression because all of the promoters were induced and saturated. Economically, 2.25 mM IPTG was determined as the optimal concentration of the inducer. *E. coli* BL21 bacteria has an acceptable growth and activity in the temperature range of 30-40 ˚C and 4-6 h of incubation after adding 2.25 mM IPTG as a bacterial inducer. The incubation time after the addition of the inducer is a key factor in the expression level of the recombinant protein. Based on the results, there was no significant difference between the maximum expression of recombinant GAD between 6 and 7 h after adding the inducer. The results showed that the best incubation time after adding the inducer for maximum expression of recombinant GAD is 6 h. The optimal temperature and time of the process to achieve the maximum expression of the recombinant GAD is 37 ˚C and 6 h (Fig. 4A).

E. coli BL21 bacteria had good growth activity in the range of pH = 6.8-7.5. Its optimal pH for the activity and expression of recombinant GAD was 7.2. With increasing temperatures up to 37 ˚C, the expression of recombinant GAD increased with a slow gradient. However, from the temperature of 37-44 ˚C, the activity of bacteria in the expression of recombinant GAD decreased significantly. In general, bacteria show more heat resistance at optimal pH. When the pH of the environment changes to acidic or alkaline, bacteria die quickly (Fig. 3B).

The culture conditions including aeration at about 120 rpm, inoculation concentrations of about 3% v/v, the temperature of 37 ˚C, pH = 7.2, and 6 h of incubation after adding 2.25 mM IPTG were the best environmental conditions for the activity of bacteria by the design expert software (Fig. 3C,D). So, the maximum expression of recombinant GAD was obtained under these conditions (Fig. 3E, Fig. 6). The results of this research were consistent with the results presented by other researchers. Shoja (2015) reported that Phycocyanin Alpha Subunit gene showed high expression 3-4 h after induction and at 37 ˚C in *E. coli* BL21. In a similar study, observed the highest concentration of recombinant Defensin expressed by *E. coli* BL21 at the optimal temperature of 30 ˚C for 6 h after induction (Mortazavi, 2014).

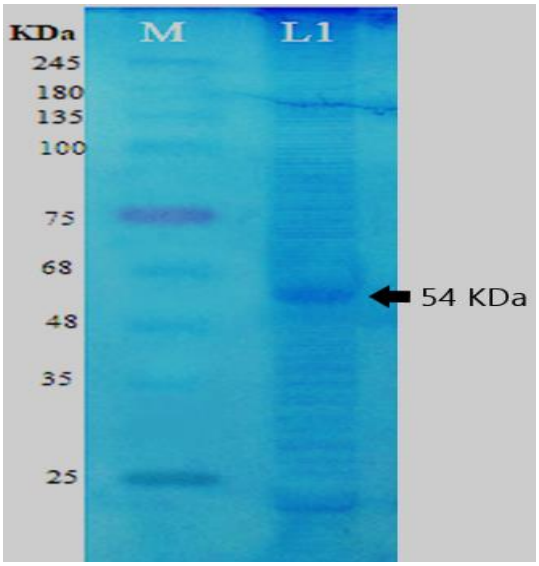


Fig. 6. Proteins expressed by *E. coli* BL21 containing PET21a (+)-GAD recombinant plasmid in optimal culture conditions (L1). M: Protein ladder.

Effect of Incubation time on recombinant GAD expression

Incubation time impacts bacterial cell growth and subsequently targets gene expression. The recombinant protein expression can be increased with optimum incubation time. This is due to the limitations in the composition of the culture medium. Therefore, optimizing cultivation conditions is crucial to maximize recombinant protein expression in engineered *E. coli* BL21 (DE3). The incubation time after the addition of the inducer had a significant effect on the expression of recombinant GAD ($P < 0.0001$), (Fig. 2B). The expression of recombinant GAD reaches its maximum level 6 h after the addition of the inducer (Figs. 3A and 4A-D). However, the expression level of recombinant GAD did not change significantly in longer times. This could be due to the lack of nutrients, the decrease in the pH of the culture medium, and the aging of the bacterial cells.

Effect of temperature on recombinant GAD expression

Incubation temperature is one of the most important factors affecting the growth and activity of bacteria. The effect of temperature on the growth of bacteria has a complex mechanism. Incubation temperature also significantly affects bacterial cell growth and protein gene expression by bacteria by changing the physical properties of the cell membrane. Therefore, the temperature is a critical factor that must be controlled to achieve maximum gene expression of the target protein. The bacterial activity also decreased sharply at temperatures higher than 40 °C (Figs. 2C, 3B, and 5A-C).

Effect of aeration on recombinant GAD expression

The rotation speed of the shaker incubator is one of the most important parameters affecting the expression of the recombinant protein (Joshi, 2008). Due to keeping the cells in suspension, it prevents from settling of cells and the formation of temperature layers. As a result, it creates a more uniform environment so that all *E. coli* bacteria cells are exposed to nutrients and oxygen (Beg, 2002; Ibrahim, 2015). Oxygen is often considered a limiting factor in a high-density cell culture. However, oxygen supply can be increased by increasing the speed of aeration or stirring (Joshi, 2008). Therefore, culture aeration is one of the influential factors affecting microbial growth and recombinant enzyme production. As shown in Fig. (2D), the growth of *E. coli* BL21 bacteria was inferior at a shaking speed of 0 rpm (static condition). Both bacterial growth and recombinant GAD production increased with increasing aeration of up to 100 rpm (Figs. 3C and 5D,E). These findings were consistent with the results reported by Joshi (2008) and Ibrahim (2015). The positive effect of aeration on *E. coli* BL21 growth and recombinant GAD expression can be related to the fact that this bacterium is highly aerobic.

Effect of different carbon sources on recombinant GAD expression

One of the important measures for the production of biological control agents on an industrial scale is to find suitable raw materials and design a commercial cultivation environment using them. One of the most important features of these materials that can be mentioned is economics and availability. In industrial-scale production, the cultivation environment should result in the highest biomass production and product quality with the lowest cost at the same time. Using wastes from food industries and agricultural by-products as sources of carbon and nitrogen due to their reasonable costs could improve the economic value of the commercial production of biological products. As shown in Fig. (7), the expression level of recombinant GAD by *E. coli* BL21 bacteria increased significantly with increasing glucose percentage up to 0.2% (w/v) in the LB broth medium. However, higher glucose concentrations did not result in any significant change in the recombinant protein expression efficiency. The result of the current research was consistent with the results announced by Sezonov (2007). Jung (2010) investigated the efficiency of cellulose production in the bacterium *Acetobacter* sp. The media containing glucose, fructose, and sucrose with concentrations (1, 2, 3, and 4% (w/v)) were examined. Their research showed that the highest and the lowest efficiencies of bacterial cellulose production were related to culture media containing glucose and sucrose, respectively. Therefore, by increasing the percentage of glucose up to 4% (w/v), the efficiency of bacterial cellulose production increased.

Using pure carbon sources such as glucose in the production of recombinant protein is very expensive and is not possible on an industrial scale. Therefore, in this research, agricultural waste and industrial by-products such as corn syrup and molasses were used as cheap resources.

We demonstrated that the expression rate of recombinant GAD by *E. coli* BL21 increased significantly with the increase of molasses concentration in the culture medium. Therefore, the highest expression rate of recombinant GAD (1.96 mg/mL) was obtained in the concentration of 5.65% (w/v) molasses (Fig. 8). The lowest level of recombinant GAD expression was observed in lower concentrations of molasses. Also, the growth rate of bacteria and the expression of recombinant GAD decreased significantly in higher concentrations of molasses (around 7%) due to the presence of a series of compounds such as heavy metals, colored compounds, and other substances (Gomaa, 2014). In molasses, the results obtained in the current research were somewhat similar to the results reported by Jung (2010). They found the highest amount of recombinant cellulose production by *Acetobacter* sp. V6 was in culture media containing 5% (w/v) sugar beet molasses. In a study of polyhydroxyalkanoate production by *Bacillus subtillis,* observed the maximum production efficiency in culture media containing 6% (w/v) molasses (Gomaa, 2014). In a similar study, on the design of the culture medium for the semi-industrial production of *Bacillus subtilis* UTB96 concluded that the maximum growth of the bacteria occurs in 10 g/L of sugarcane molasses culture medium (Ghasemi, 2014). Mousavi Nasab (2010) obtained the highest concentration of microbial production of lysine by *Brevibacterium linens* in a culture medium containing 10 g/L of molasses. The results of their research showed that with the increase in the concentration of molasses in the culture medium, the amount of lysine production decreased.

The results showed that *E. coli* BL21 expressed almost the same amount of recombinant protein by replacing 65.5% (w/v) of molasses instead of pure glucose. Considering the high volume of molasses produced in sugar factories and the results obtained from this research, molasses, as a reasonable and available carbon source, could be a suitable option for the cost-effective industrial production of recombinant GAD. Sugar beet molasses improves the growth and activity of bacteria due to the presence of nitrogen and sulfur content. Although, it should be noted that molasses contains some heavy metals, colored compounds, and various other substances. These disturbing compounds cannot be completely removed even by acid treatment and heat, and in high concentrations of molasses, they prevent the growth and activity of bacteria. Therefore, utilizing these industrial wastes, in addition to reducing environmental pollution and economic profitability for sugar factories, causes the production of cheap recombinant protein.

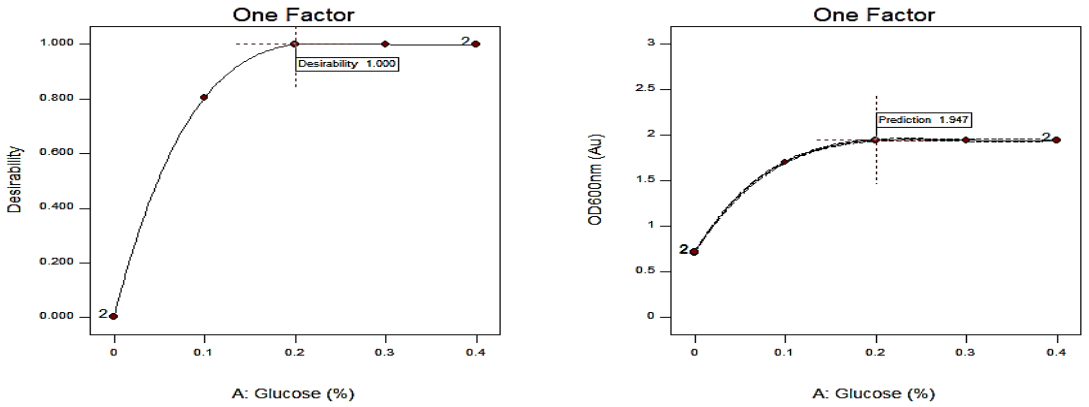


Fig. 7. The effect of different concentrations of glucose on the expression of recombinant GAD.

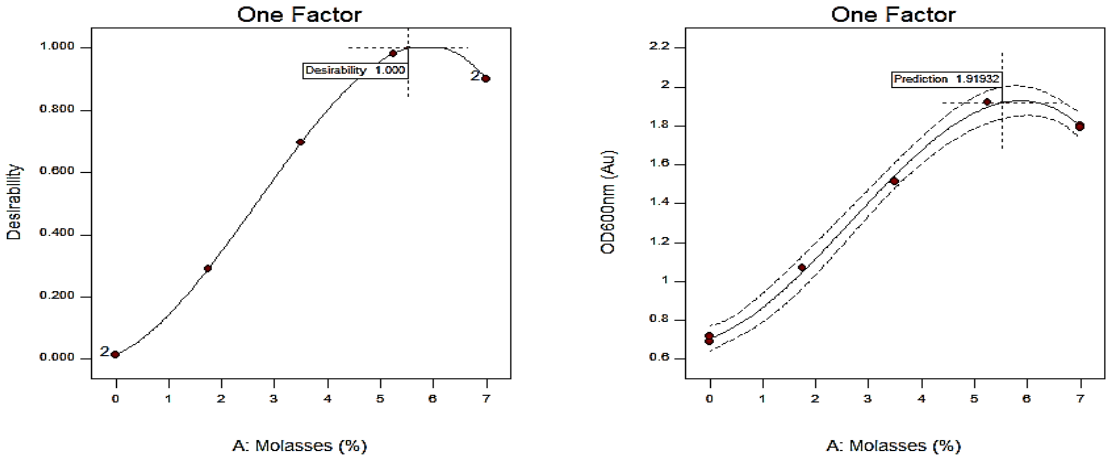


Fig. 8. The effect of different concentrations of sugar beet molasses on the expression of recombinant GAD by *E. coli* BL21.

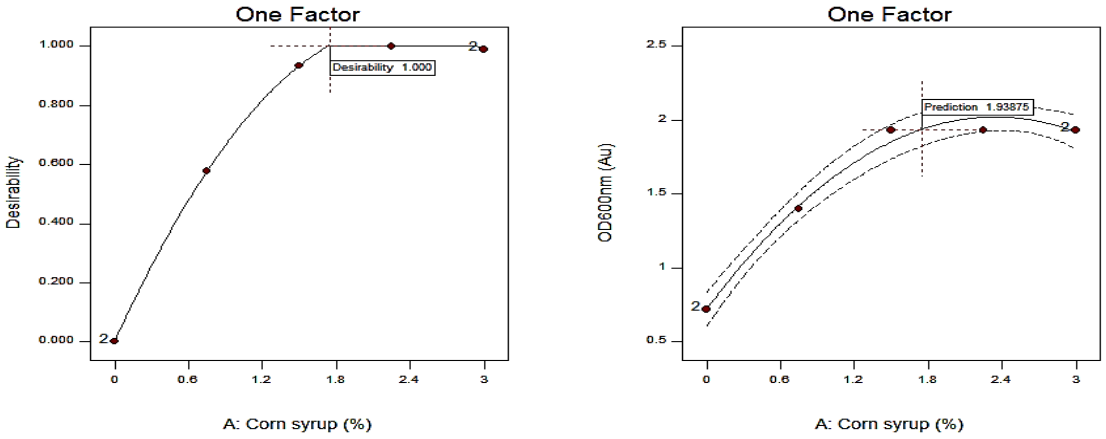


Fig. 9. The effect of different concentrations of corn syrup on the expression level of recombinant GAD produced in *E. coli* BL21.

As shown in Fig. (9), the expression rate of recombinant GAD by recombinant *E. coli* BL21 increased significantly by increasing the concentration of corn syrup up to 1.5% (w/v) in the LB broth culture medium. This is due to the availability of sugar required by the bacteria. Light absorption at 600 nm in culture medium without carbon source and medium containing 0.75% (w/v) corn syrup was 0.714 and 1.310, respectively. Light absorption reached its maximum value in these experiments (OD_{600nm} = 1.934) with the increase of corn syrup concentration up to 1.5% (w/v). However, an increase in the expression of recombinant GAD was not observed at concentrations higher than 1.5% (w/v) of corn syrup. Based on the final results of optimization and validation of the model, the recombinant *E. coli* BL21 bacterium expresses almost the same amount of the recombinant GAD in the culture medium containing 1.8% (w/v) corn syrup compared to the culture medium containing 0.2% (w/v) of glucose. Ghasemi (2014) in designing the culture medium for the semi-industrial production of *Bacillus subtilis* UTB96 showed that in the LB culture medium containing corn syrup with a concentration of 2 g/L, the bacteria had the maximum growth. In similar research, showed that the medium containing 5.5 and 6.15% (w/v) of corn steep liquor were the optimum production points of biomass and endoglucanase enzyme in methylophilic yeast *Hansenula polymorpha*, respectively (Jafarimanesh, 2017).

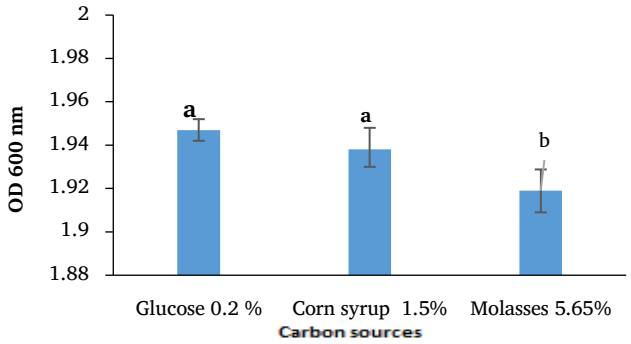


Fig. 10. Light absorption in optimal mediums.

The result showed that the recombinant *E. coli* BL21 grown better in the medium containing glucose and has the highest expression efficiency of the recombinant GAD. The expression rate of recombinant GAD by recombinant *E. coli* BL21 was significantly higher in an LB culture medium containing corn syrup than in a culture medium containing molasses. This is due to the high percentage of glucose in corn syrup compared to molasses and also because of the higher purity grade of corn syrup compared to molasses. Based on the obtained results, the highest expression efficiency of recombinant GAD according to the type of carbon source of the culture medium is as follows:

Glucose > corn syrup > molasses

Based on the results of the present research, 0.2% glucose (w/v) was the optimal carbon source in the laboratory scale for the growth and activity of *E. coli* BL21. However, it is possible to use 1.5% (w/v) corn syrup or 5.65% (w/v) molasses on an industrial scale as a cost-effective and available carbon source to reach a similar level of *E. coli* BL21 activity (Figs. 10 and 11). The concentration of purified recombinant GAD in carbon sources of 0.2% glucose, 1.5% corn syrup and 5.65% molasses was 2.155, 2.07 and 1.96 mg/mL, respectively.

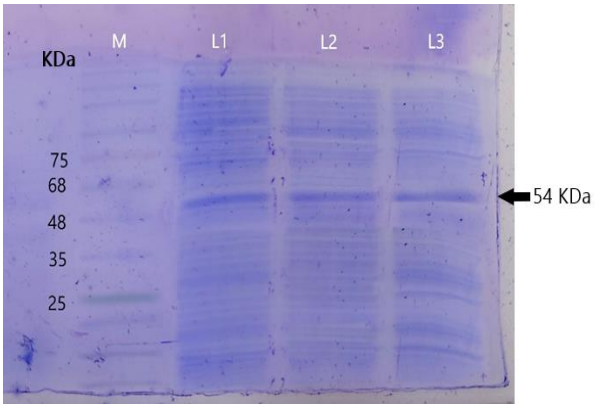


Fig. 11. The expression rate of recombinant GAD in culture media containing L1: 0.2% glucose (w/v), L2: 1.5% corn syrup (w/v), and L3: 5.65% molasses (w/v) v), M: protein marker.

Enzyme activity determination

Observing the Ruhemann's purple band on the TLC plate showed that the recombinant GAD enzyme was active and could convert glutamate to GABA through the decarboxylation reaction. Ninhydrin causes the release of amino and carboxyl groups. Amino acid is attached to ninhydrin through the free electron pair of amine nitrogen. Then, the amino acid is converted into an aldehyde compound by removing a carbon dioxide molecule and connecting the carboxyl group and decarboxylation. Ninhydrin is converted into dantin hydrin and a Ruhemann's purple complex is formed. Thin layer chromatography has been used by different researchers to evaluate GABA production (Ko, 2013; Li, 2009; Tajabadi, 2015; Zareie, 2019). The amount of GABA produced using recombinant GAD produced in culture media containing 0.2% glucose, 1.5% corn syrup and 5.65% molasses was 23.22, 22.96 and 22.35 mg/mL, respectively (Fig. 12).

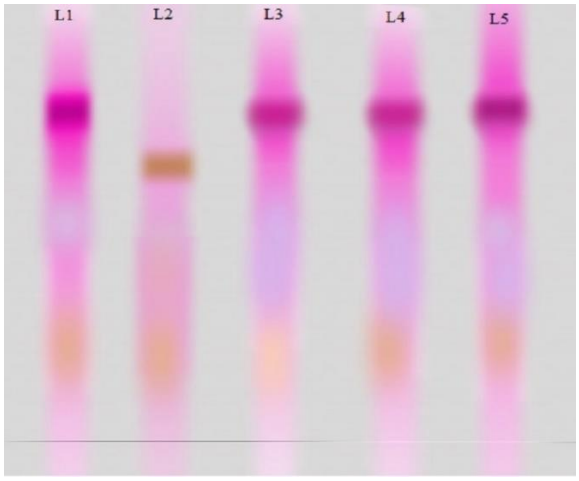


Fig. 12. TLC plate of L1: standard GABA (1000 mg/L), L2: monosodium glutamate, GABA produced by the recombinant GAD produced in culture media containing L3: 5.65% molasses (w/v) v), L4: 1.5% corn syrup (w/v), L5: 0.2% glucose (w/v).

Conclusion

According to the research results, the recombinant *E. coli* BL21 (DE) grew better in the culture medium containing glucose and had the highest expression efficiency of the recombinant GAD, probably due to the rapid absorption of this carbohydrate by the bacterial cell. This has resulted in a higher bacterial cell growth rate and maximum expression of the recombinant GAD gene. In general, using reasonable and available carbon sources (such as corn syrup and sugar beet molasses) reduces the cost of recombinant enzyme production. Molasses is suitable for the growth of microorganisms because it contains mineral ions, other nutrients, and carbohydrates. This is cost-effective in the industrial production of recombinant enzymes. The advantages of using industrial wastes such as molasses include economic profitability for factories by converting by-products into value-added products and producing a large amount of reasonably priced recombinant GAD. In this study, we produced recombinant GAD on an industrial scale to produce GABA in large volumes. With this method, the global demand for GABA in the food and pharmaceutical industries can be met.

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Author contributions

Hanieh Yarabbi: Presenting the research idea and study design, Data collected, Data analysis, Data analysis and

interpretation, Revising and editing the manuscript, Supervising the study, Approval of the final version; **Sahar Roshanak**: Data collected, Data analysis, Data analysis and interpretation, Revising and editing the manuscript, Supervising the study, Approval of the final version; **Seyed Ali Mortazavi**: Presenting the research idea and study design, Data analysis and interpretation, Supervising the study, Approval of the final version; **Masoud Yavarmanesh**: Presenting the research idea and study design, Data analysis and interpretation, Revising and editing the manuscript, Supervising the study, Approval of the final version; **Ali Javadmanesh**: Presenting the research idea and study design, Data analysis and interpretation, Supervising the study, Approval of the final version.

Conflict of Interest

The authors declare that they have no conflict of interest.

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