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Research Article

Enhancement of Alpha 1-antitrypsin Production in *Pichia pastoris* by Designing and Optimizing Medium Using Elemental Analysis

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Background: Human alpha 1-antitrypsin (AAT) is a monomeric glycosylated protein; it is the potent inhibitor of a whole range of serine proteases and protects tissues against their destructive eff ects. The human plasma-derived AAT, which is currently used to augment the AAT level in patients, is limited due to high cost and source limitation. Recombinant production of AAT can be considered as a potential alternative.
Objectives: This study aims to develop and optimize a new chemically defi ned medium based on an elemental analysis of the yeast *Pichia pastoris* for an effi cient culture of the recombinant yeast-producing secretory AAT.
Materials and Methods: An elemental analysis of Carbon (C), Hydrogen (H), Nitrogen (N), Sulfur (S); CHNS in its abbreviated form, and metallic elements was performed to determine the exact molecular constituent of the *P. pastoris*. The medium components were selected according to the obtained formula; they were optimized by the response surface methodology (RSM). The grown yeast cell was measured at the end of 18 h glycerol batch culture. The amounts of AAT production and elastase inhibitory capacity (EIC) were measured at the end of three days' methanol feeding.
Results: The optimized medium compositions consist of glycerol (40 $g.L^{-1}$), KH_2PO_4 (24.78 $g.L^{-1}$), NaCl, (0.88 $g.L^{-1}$), $MgSO_4.7H_2O$ (1.95 $g.L^{-1}$), $(NH_4)_2SO_4$ (22.76 $g.L^{-1}$), and trace elements (20 $mL.L^{-1}$). The presented quadratic models show that KH_2PO_4 and $(NH4)_2SO_4$, are the most abundant ones in the *P. pastoris* biomass and have the greatest eff ect on the cell growth, EIC, and AAT protein production responses.
Conclusions: According to the results of this study, it can be concluded that the characterizing cell composition formula could be considered as an appropriate method to design culture media in order to improve cell growth and productivity. Compared to the common *P. pastoris* chemically defi ned media, FM22 and BSM, production of AAT protein increased by 1.5 and 1.4 times, respectively, in this new medium.

Keywords: Alpha 1-antitrypsin, Designing Medium, Elemental Analysis, Medium Optimization, *Pichia pastoris.*

1. Background

AAT protein is the most abundant protease inhibitor in human circulation; it is mainly produced in the liver and secreted to the bloodstream (1,2). This protein is introduced as the archetype of the serpin protease inhibitor (serpin) superfamily (3). AAT is a potent inhibitor of the multiple serine proteases, including elastase, trypsin, and proteinase 3, as well as some non-serine proteases such as aggrecanase and metalloprotease MMP9; it balances the action of substrate proteases in different tissues (mainly neutrophil proteases in the lung) (4). The average level of AAT in the blood is 1.3 $mg.mL^{-1}$. Individuals with AAT levels less than 15% of the normal are considered AAT-deficient. They are likely to develop panacinar emphysema or chronic obstructive pulmonary disease (COPD) at young ages (2, 3, 5). AAT protein augmentation is the only specific therapy for lung diseases related to the AAT deficiency. For this purpose, purified AAT protein is administered by a weekly intravenous infusion throughout the patient's life to boost the AAT level in the blood. All the currently available commercial AAT proteins are

derived from human plasma. Despite being effective, this drug suffers from the considerable limitations that include the risk of viral infection, source limitation, and high cost for patients and their families (6, 7). The production of the recombinant AAT can be considered a notable solution for these problems. Hence, different studies have shown the production of AAT in different hosts;from bacteria to animal cells (8-15).

The Methylotrophic yeast *P. pastoris* is an attractive host for the production of the many human glycoproteins like AAT. This host benefits from advantages such as rapid growth to very high cell densities, presence of tightly regulated alcohol oxidase 1 (AOX1) promoter, efficient secretion machinery system, ease of genetic manipulation, and the capacity ofintroducing many posttranslational modifications (13, 16).

The level of expression of a heterologous protein in a host depends on multiple variables specific to the protein's characteristics and the host. Such variables should be carefully evaluated during the development of a bioprocess. Different techniques for optimizing the gene copy number, codon preference, enhancing mRNA stability, promoter selection, using fusion proteins, and many other gene manipulation practices have been explored to improve the process of producing different heterologous proteins in the yeasts. Optimization the yeast culture conditions and the media compositions are another traditional techniques to achieve a high cell density for the high-level of production, as well as for improving the stability of the recombinant protein, and consequently maximizing the profitability.

The culture medium is a key player when a high cell density for any microorganism is desired. The most common growth culture media for achieving a high cell density for *P. pastoris* are the basal salt medium (BSM) proposed by Invitrogen Co, FM22 formulated by Stratton *et al.,* and the medium reported by D'Anjou and Daugulis. BSM and FM22 media generally provide a high concentration of the basic elements, while the D'Anjou medium has a low concentration of the chemical elements and thus is associated with some problems including precipitation, imbalanced composition, and undesirable ionic strength (16, 17). Nitrogen source, which is one of the most important elements to formulate a medium, in BSM and FM22 is also employed to adjust the pH. Hence, nitrogen starvation may occur during the high cell density cultivation of the *P. pastoris,* which would directly increase proteolysis events and degradation of the extracellular proteins. On the other hand, it is necessary to avoid its accumulation because it can provoke the inhibition of the growth and enlarge the lag phase (16). To reduce the problems associated with the current media, it is important to design and optimize a new medium for the biomass generation and an efficient production of the recombinant *P. pastoris*.The necessary amount of each nutrient source is variable for the different microorganisms; it should be calculated on the basis of the mass balance for the cell growth and product formation (18). Most studies do not address the design of the culture medium and only deal with the optimization of the existing successful culture medium.

2. Objectives

This study aims to formulate a new culture medium by using an elemental analysis of *P. pastoris* cells. It also aims to optimize each component to reach the high cell density of the recombinant *P. pastoris* and to maximize the secretory production of the AAT by using the RSM.

3. Materials and Methods

3.1. Microorganism, Media, and Culture Conditions

Recombinant *P. pastoris*, which expresses AAT under the control of the AOX1 promoter with the Mut^+ phenotype was obtained from the previous study (8).
The primary culture medium used for the cultivation of the *P. pastoris* for CHNS and the elemental analysis of the cells was YPG (yeast extract (10 $g.L^{-1}$), peptone (20 $g.L^{-1}$) and glycerol (20 $g.L^{-1}$)). Other culture media used for the optimization of the medium, AAT production quantity, and function assays were obtained in accordance with the experimental design explained in the following sections. Yeast cells were cultivated at 200 RPM and 30 ºC.

All experiments were performed in 250 mL Erlenmeyer flasks with 50 mL of working mediumand 40 $g.L^{-1}$ of the glycerol as a carbon and energy source. Inoculation into the flasks was done in such a way that the initial optical density at 600nm was the same for all experiments.

3.2. Induction of AAT Production

After 20 h of cultivation in YPG, the culture was centrifuged for 5 min at 5000 ×g and the medium was substituted with a new inductive one containing 0.5% of the pure methanol to initiate AAT production. The inductive condition was maintained for 72h with the addition of 1% methanol every 24 h (19-21).

3.3. Elemental Analysis

For CHNS analysis, the yeast cells at the end of the log phase of the growth of the YPG medium was separated and dried at 80 ºC for 18 h. The C, H, N, and S elemental analysis of the yeast biomass was performed on an

Elemental CHNS Analyzer model, Vario EL III (Varion EL, Elementar Analyzer system GmbH, Germany).

The composition of the mineral elements was determined using SPECRTO ARCOS ICP-OES (SPECTRO, Germany). For this purpose, fresh yeast biomass was digested with aqua regia (nitric acid/ hydrogen chloride [1:3]) for 45 min at 80 ºC. The digested solution was passed through the cellulose acetate membrane filters (0.45 µm) before being analyzed by ICP-OES.

3.4. Determination of Yeast Biomass

The yeast biomass was determined by OD_{600} measurements using the BiochromUltrospec 60 spectrophotometer (1 OD_{600} unit is equivalent to 0.25 g $DCW.L^{-1}$), data were not shown).

3.5. Assessment of AAT Activity

The activity of the produced recombinant AAT in the different media was determined by the elastase inhibition assay according to the previously described method (22) with slight modifications. N-Succinyl-Ala (3)-*ρ*-nitroanilide (S4760, Sigma) was used as an elastase substrate. As much as 100 µL of the each culture supernatant was dissolved in 0.2 M Tris-HCl buffer (pH 8.0) and was incubated with 0.01 (Unit. mL^{-1}) elastase (Cat #45124, Sigma) for 5 min, before adding 0.07 ($mg.mL^{-1}$) substrate in the final volume of 300 µL. The substrate hydrolysis and the release of *p*-nitroaniline were measured by the spectrophotometer at 410 nm after 15 min at 25°C. EIC was expressed as a percentage of the enzyme activity inhibited by the recombinant AAT compared with the blank vial (without AAT).

3.6. AAT Quantification

The secreted AAT was determined at the end of each experiment by using the human Serpin A1 DuoSet Kit (DY1268, R&D Systems), according to the manufacturer's instructions. All the samples were diluted equally in the diluents buffer (0.05% (v/v) Tween 20 in PBS, pH 7.2-7.4) and assayed in triplicates. The Recombinant Human Serpin A1 was applied as the standard sample.

3.7. RSM Experimental Design and Statistical Analysis

A central composite design (CCD) was used in the optimization of the culture medium components for maximizing AAT production. The concentrations of [$g.L^{-1}$] (KH_2PO_4, NaCl, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and trace elements solution ($mL.L^{-1}$) in the medium were independent variables optimized at five different levels (-α, -1, 0, +1, +α). As dependent variables, biomass production at the end of glycerol batch phase, EIC results, and the quantity of the produced AAT at the end of the methanol-feeding step was considered as responses. A total of 50 experimental runs, including eight replicates for the centre point, were performed. The role of each variable and their interaction is explained by applying the following quadratic Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_i \sum_j \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where k, $\beta\beta_0$, $\beta\ \beta_i$, x_i, $\beta\beta_{ii}$, $\beta\ \beta_{ij}$and $\varepsilon\ \varepsilon$ represent the number of variables, the constant term, coefficients of the linear parameters, variables, coefficients of the quadratic parameters, coefficients of the interaction parameters, and residuals associated with the experiments, respectively.

4. Results

4.1. Elemental Composition of P. pastoris and Medium Formulation

To formulate a new appropriate medium, the components concentration should be optimized based on cell composition, growth stoichiometry, product formation, yield coefficient, and other parameters. Medium culture composition must supply the required energy to support cells' maintenance as well as cell growth and metabolite production. At the first step, the equation (Eq. 2) is written on the basis of the mass balance for cell growth and product formation.

Carbon+ energy + Nitrogen + O2 + Other requirements → Biomass + Products + CO2 + H2O + Heat (2)

Quantitative calculation of the above formula is a helpful method to minimize the surplus or shortage of the culture medium ingredients during the fermentation process. Therefore, the concentration range of such components was determined based on the minimum amount needed for the optimal biomass production. The required substrates' concentrations can be estimated by knowing how much biomass is needed for the product formation. In this study, to design a new efficient chemically defined media, the macro and micro elemental compositions of the *P. pastoris* yeast cells were determined by using two elemental analysis techniques, namely CHNS and ICP-OES analysis. These two analytical methods are excellent tools for providing the elemental composition of different cells and determining their empirical formula (23, 24).

The CHNS analysis was used to quantify the mass

Table1. CHNS analysis of the dried *P. pastoris* material.

Element name	Weight (mg)	Weight (%)
Nitrogen	0.72	7.9
Carbon	4.09	44.6
Hydrogen	0.59	6.5
Sulfur	0.038	0.42

fraction and determination of the ratio of main macro elements; carbon, nitrogen, hydrogen, and sulfur (Table 1). The ICP–OES analysis was used to determine the mass fraction of the trace elements including Mg, S, K, Ca, Na, Fe, P, Co, Zn, Cu, Pb, and Mn (Table 2). The quantities of the shared sulfur elements in the two analyses were used to link their results and to determine the *P. pastoris* chemical formula, while the oxygen was determined by the difference. The ultimate formula of *P. pastoris,* based on the ICP and CHNS analysis, is shown below:

$$CH_{1.7}N_{0.15}O_{0.55}S_{0.0032}K_{0.038}P_{0.022}Na_{0.0012}Mg_{0.001}$$

According to this chemical formula, the molar weight of *P. pastoris* is about 30 g; therefore, 3.3 mol of *P. pastoris* is needed to produce 100 g of biomass dry weight (DW). Based on these analyses, the quantities of the components for obtaining 100 g of DCW *P. pastoris* and related medium optimization by the RSM methodology is shown in Table 3.

According to the literature, mineral salts of $(NH_4)_2SO_4$, NaCl, $MgSO_4.7H_2O$, and KH_2PO_4 were selected to supply nitrogen, phosphorus, sulfur, potassium, magnesium, and sodium elements (25).

A new trace metal elements solution ($FeSO_4.7H_2O$ (12 g.L^{-1}), $ZnCl_2$ (13 g.L^{-1}), $CuSO_4.5H_2O$ (5 g.L^{-1}), $CaCl_2$ (0.9 g.L^{-1}), $MnSO_4.H_2O$ (4.5 g.L^{-1}), and 5 mL H_2SO_4) was formulated according to the obtained *P. pastoris* formula and then analyzed applying RSM to find the best quantity of this solution in the culture medium for the selected responses.

4.2. Experimental Design and Statistical Analysis

Using the CCD–RSM methodology, 32 factorial points, 10 axial points, and eight central point experiments were designed to obtain the optimum quantity of the medium compositions and to optimize the culture medium. The experimental design and responses (the optical density of the culture media at 600 nm, the percentage of elastase inhibition, and production quantity of ATT) are indicated in Table 4. The analysis of variance (ANOVA) was used to evaluate the effect of each factor at OD_{600}, elastase inhibitory capacity, and AAT production. The ANOVA Table in all the three responses shows that the results were very similar. To avoid repetition, the results were reported based on the production of the AAT. According to the ANOVA (Table 5), a quadratic model is appropriate for explaining the effect of the culture media components on the responses. This quadratic model is shown in Eq. 3, where A, B, C, D, and E are independent variables.

$$Production\ of\ AAT = 6.99 + 1.51A - 0.14B - 0.36C + 0.72D + 0.17E - 0.26AB + 0.28AD \quad (3)$$

Table 2. The elemental composition of the *P. pastoris by* analysis of ICP-OES.

Element	Amount (g.Kg^{-1} DCW)	Element	Amount (g.Kg^{-1} DCW)
Sulfur	4	Copper	0.1
Magnesium	1.25	Iron	0.2
Potassium	29.2	Manganese	0.07
Sodium	1.87	Zinc	0.25
Phosphorus	25.7	Calcium	0.82
Lead	0.0023	Cobalt	0.005

Table 3. Factors and their levels in the central composite design for a medium culture optimization.

Chemicals	Symbol	Required concentration	-α	-1	0	1	A
KH_2PO_4	A	19.2 g.L^{-1}	12.00	17.21	21.00	24.78	30.00
NaCl	B	0.9 g.L^{-1}	0.50	0.79	1.00	1.21	1.50
$MgSO_4.7H_2O$	C	2 g.L^{-1}	1.50	1.93	2.25	2.56	3.00
$(NH4)_2SO_4$	D	12 g.L^{-1}	5.00	12.24	17.50	22.76	30.00
Trace element	E	20 mL.L^{-1}	10.00	15.80	20.00	24.20	30.00

Table 4. Levels of the variables and CCD for production quantity of AAT.

RUN	KH_2PO_4 $g.L^{-1}$	NaCl $g.L^{-1}$	$MgSO_4.7H_2O$ $g.L^{-1}$	$(NH_4)_2SO_4$ $g.L^{-1}$	Trace Element $mL.L^{-1}$	Responses		
						Optical Density	EIC %	AAT Production ($mg.L^{-1}$)
1	24.8	0.79	2.57	12.24	15.8	9.6	60	6.4
2	17.2	0.79	1.93	12.24	24.2	8.6	46	5.4
3	24.8	1.21	2.57	12.24	24.2	9.6	65	8.5
4	24.8	0.79	1.93	12.24	24.2	9.6	64	9.5
5	17.2	1.21	2.57	22.76	15.8	10.5	47	5.2
6	17.2	0.79	2.57	12.24	24.2	8.5	43	5.3
7	21	1	3	17.5	20	11.2	65	6.3
8	17.2	0.79	2.57	22.76	15.8	10.5	58	6.2
9	21	1.5	2.25	17.5	20	10.6	60	6.6
10	12	1	2.25	17.5	20	9.6	35	3.2
11	24.8	0.79	2.57	12.24	24.2	9.6	64	7.7
12	24.8	0.79	2.57	22.76	24.2	10.5	62	8.3
13	24.8	0.79	1.93	12.24	15.8	9.6	67	9.3
14	24.8	1.21	1.93	12.24	15.8	9.6	56	6.5
15	21	1	2.25	17.5	20	12	58	6.6
16	21	1	2.25	17.5	20	12.2	62	7.7
17	17.2	1.21	2.57	12.24	24.2	8.6	45	5.3
18	17.2	1.21	1.93	22.76	24.2	10.5	51	6.3
19	24.8	1.21	2.57	12.24	15.8	9.6	54	6
20	21	1	2.25	17.5	30	11.2	56	7
21	17.2	0.79	2.57	12.24	15.8	8.6	39	4.2
22	21	1	2.25	17.5	20	12.2	60	7.1
23	21	1	2.25	17.5	20	12.2	64	8.9
24	24.8	0.79	2.57	22.76	15.8	11.6	67	9.2
25	21	1	2.25	5	20	6.6	48	5.4
26	21	0.5	2.25	17.5	20	10.6	52	6.8
27	17.2	1.21	2.57	12.24	15.8	8.6	48	4.9
28	24.8	1.21	1.93	22.76	24.2	10.5	69	9.9
29	21	1	2.25	17.5	20	12.2	62	7.6
30	24.8	1.21	1.93	22.76	15.8	10.5	67	9.7
31	17.2	1.21	1.93	22.76	15.8	10.5	58	6.7
32	17.2	0.79	1.93	12.24	15.8	8.6	38	4.6
33	17.2	0.79	1.93	22.76	15.8	10.5	50	5.5
34	17.2	1.21	1.93	12.24	15.8	8.6	46	4.7
35	21	1	2.25	17.5	10	11.2	62	7.3
36	24.8	1.21	1.93	12.24	24.2	9.6	52	6.8
37	24.8	0.79	1.93	22.76	24.2	11.6	71	10.8
38	24.8	1.21	2.57	22.76	15.8	11.6	52	7.6
39	17.2	0.79	1.93	22.76	24.2	10.5	55	5.8
40	17.2	1.21	1.93	12.24	24.2	8.6	46	5.5
41	30	1	2.25	17.5	20	11.6	65	9.4
42	24.8	0.79	1.93	22.76	15.8	11.6	67	10.5
43	21	1	1.5	17.5	20	11.2	51	7.5
44	21	1	2.25	17.5	20	12.2	60	6.7
45	21	1	2.25	17.5	20	12.2	60	6.9
46	21	1	2.25	30	20	10.4	66	9.6
47	24.8	1.21	2.57	22.76	24.2	10.5	68	9.8
48	17.2	1.21	2.57	22.76	24.2	10.5	32	5
49	21	1	2.25	17.5	20	12.2	58	6.8
50	17.2	0.79	2.57	22.76	24.2	10.6	50	5.2

Table 5. ANOVA analysis for the response surface model.

Source	Sum of squares	DF	Mean squares	F-Value	P-Value
Model	141.82	20	7.09	12.99	<0.0001
KH_2PO_4	98.92	1	98.92	181.25	<0.0001
NaCl	0.82	1	0.82	1.51	0.21
$MgSO_4.7H_2O$	5.6	1	5.6	10.26	0.0033
$(NH4)_2SO_4$	22.31	1	22.31	40.88	<0.0001
Trace element	1.19	1	1.19	2.18	0.15

The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.93, indicating that 93% of the variability in the response could be explained by the model. The relatively high value of R^2 indicates that the quadratic model fits the data under these experimental conditions. The model's *F*-Value of 12.96 implies that the model was significant. The significance of each component was evaluated by using P-value, which was also very low (P <0.0001), thus indicating the significance of the model (26-28). The response surface curve was plotted in order to determine the optimum level of each variable for the maximum response. The response surface curves are shown in Figures 1 and 2. Each Figure demonstrates the effect of the two factors, while the other factors are fixed at the middle level.

4.3. Optimized Medium and Validation of Experimental Model

The model predicted the optimal values with the desirability of 96%, where the values were KH_2PO_4 (24.78 g.L^{-1}), NaCl, (0.88 g.L^{-1}), $MgSO_4.7H_2O$ (1.95 g.L^{-1}), $(NH_4)_2SO_4$ (22.76 g.L^{-1}) and trace element (20 mL.L^{-1}). A verification experiment was performed by using the predicted optimum medium composition, while the AAT production was measured to be 10.3 (mg.L^{-1}) very close to the predicted response 10.6 (mg.L^{-1}). This amount of production was 1.5 and 1.4 times higher than the current chemically defined media FM22 and BSM, respectively (data was not shown). Hence, the validity of the proposed quadratic model and the success of the optimization are approved.

5. Discussion

The components of the medium can significantly affect cell growth, yield, and volumetric productivity, and hence it is critically important to design an optimized medium. Designing the medium is a challenging, laborious, and time-consuming process comprising many experiments. However, the new media is frequently designed in the industry and the research lab because new strains are continuously being introduced and optimization of the existing media for the established strains can be very beneficial commercially. There are many techniques for designing and optimizing media including literature search, component swapping, one at a time, biological mimicry, statistical, and mathematical techniques like an experimental

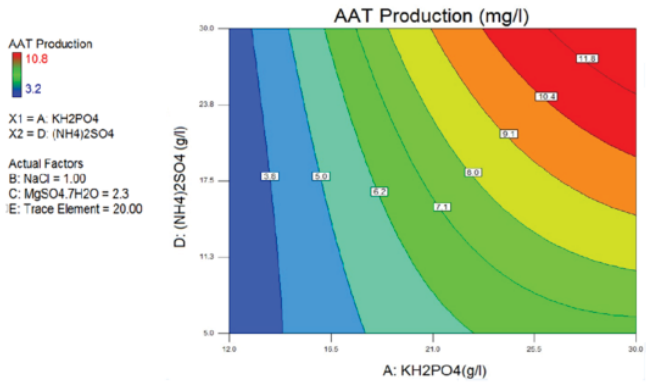

Figure 1. Contour plot for the effect of $(NH_4)_2SO_4$ and KH_2PO_4 on the production quantity of AAT.

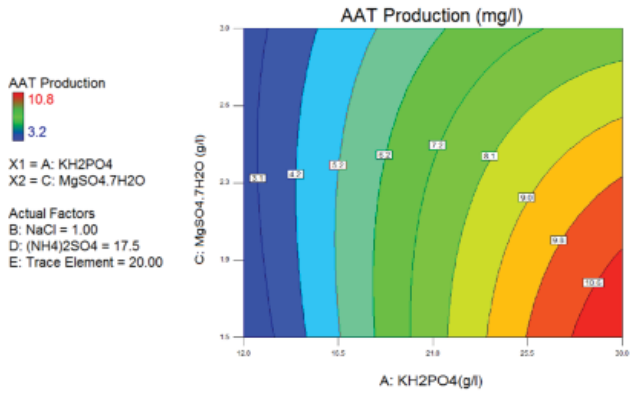

Figure 2. Contour plot for the effect of $MgSO_4.7H_2O$ and KH_2PO_4 on the production quantity of AAT.

design. Here, we used a combination of the biological mimicry and experimental design to get better results (29). The biological mimicry, also known as 'match and win strategy', is a close-ended system that is based on the concept that the medium provides the best growth and productivity while containing the right proportion of everything that the cells need. In this method, the elemental composition analysis of the host cell and desired product concentration is required. We considered this strategy as a good starting point for designing a new efficient medium. To the best of our knowledge, this is the first time that the strategy is used to formulate a new medium for the growth and production of the *P. pastoris*.

The ANOVA results and the mathematical model indicate that KH_2PO_4 and $(NH_4)_2SO_4$ have the most positive effect on the AAT production quantity. The positive effect of these components could be attributed to their elemental compositions. The interaction effect between the factors was tested by using contour plots for the possible combinations of the factors while keeping other factors constant at the middle level. KH_2PO_4 and $(NH_4)_2SO_4$ contain elements of nitrogen, hydrogen, and sulfur, which are the most abundant elements after carbon, in *P. pastoris.* These results are in agreement with the CHNS and ICP–OES analyses (30, 31). Increasing $MgSO_4.7H_2O$ has a slight negative effect on the quantity of AAT production. Magnesium is the most abundant intracellular divalent cation in all living cells, including yeasts, and plays numerous structural and functional roles in the yeast cell physiology. This metal acts as the essential cofactor for a wide range of enzymes that are involved in different metabolic and bioenergetics pathways and are critical for the production of the proteins (32). However, it has been known that high concentrations of magnesium ion stabilize ribosome-bound tRNAs and result in ribosome stalling and slow translation rate along with the protein biosynthesis (33). This fact could be an explanation for our case, which needs further study.

Any change in the NaCl concentrations within the selected range has no effect on the AAT production. These results were exactly equivalent to the cell growth (34). Compared with the other trace metal elements solutions which were introduced for the *P. pastoris* so far, the presented trace metal solution formulated in accordance with the *P. pastoris* formula is simpler with fewer components. Such simplicity has several benefits such as the lack of precipitation problem, prevention of the possible interference with the excess metals in the downstream processing, and reduction of the metal waste disposals for the sake of environmental protection (26, 35).

6. Conclusion

In this study, a unique and new culture medium has been developed for *P. pastoris* to enhance its growth and productivity. An elemental analysis strategy was used as the starting point to select the culture medium components according to the cell formulation. Next, the RSM methodology was successfully used to investigate the component interaction effects and obtaining an optimized concentration of the each elemental constituent. Thus, a newly promoted medium has been introduced with the considerable advantages including fewer components and easy preparation, lack of precipitation problem, no need for acid or base to adjust the initial pH, no heavy metal elements in the trace elements solution. Compared to the common chemically defined media for *P. pastoris*, namely FM22 and BSM, the production of AAT protein increased by 1.5 and 1.4 times respectively in this new medium. According to the results of the presented study, it can be concluded that the characterizing cell composition formula could be considered as an appropriate method for designing culture media in order to improve cell growth and productivity.

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