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Statistical optimization of α-amylase production by *Escherichia coli*using extruded bean as nitrogen and carbon source

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***Abstract*—** *Response surface methodology based on mixture design was employed for statistical optimization of medium components for the growth and production of α-amylase by Escherichia coli pAC92. The combined effects of media constituents (peptone, yeast extract and extruded beans) were analyzed using a cubic model, which was developed by3-factor simplex lattice mixture design in predicting the optimum yield of growth and α-amylase activity. Results evidenced that extruded common bean was more effective as a nutrient source for E.colipAC92 growth. On the other hand, the completely substituted medium with extruded common bean resulted in 68% of increase in the growth of Escherichia coli pAC92. In addition, the culture medium containing 0.5% of extruded bean and 0.5% of peptone reached a α-amylase activity of 44.59 U. The optimal medium composition was determined by a numerical method based on desirability function, by which the optimal composition for maximum optical density and enzyme activity was found using 0.5% peptone and 0.5% extruded common bean as media constituents. Therefore, these results evidenced that extruded common bean can be successfully used as substitute of peptone and yeast extract in culture media for production of α-amylase byE.colipAC92.*

***Keywords*— extruded bean; mixture design; α-amylase production; culture medium.**

I. INTRODUCTION

Amylases are enzymes widely used in biotechnology processes, constituting a class of industrial enzymes having ¼ of the world enzyme market. α-Amylases have potential application in food, fermentation, textile, paper, detergent, and pharmaceutical industries. In addition, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the brewing and distilling industries(Souza and Magalhães, 2010; Gashtasbi et al., 2014).

In the recent decades, the demand for industrial α-amylase has been on the rise because of their economic and environmental benefits(Hellmuth and Van-Den-Brink, 2013; Kim et al., 2014). In addition, due to rising prices of nitrogen and carbon sources, the bio-based enzymes production has been emerged as a promissory technology in biotechnology field. It is known that the development of a bio-based industrial enzymes production requires a low-cost medium and a versatile producing organism able to utilize a wide range of low-cost feedstock(Carneiro et al., 2013). In this scenario, hardened beans, an agro-industrial by-product that contains high amount of carbohydrates and proteins could be a promising nutrient source for α-amylase production.

However, the microbial production of amylases can be affected by certain factors, including microbial strain, culture medium formula and physicochemical conditions(Hii et al., 2012). Considering the composition of culture medium, the nature of nitrogen and carbon source could influence the rate of amylase production and, therefore the enhancement of target enzyme productivity in any fermentation system could be achieved through the improvement of the culture medium composition(Ye et al., 2010; Hortsch and Weuster-Botz, 2011; Boumba et al., 2013; Carneiro et al., 2013).

Conventional change one-factor-at-a-time approach has been applied to optimize the enzyme production using different medium constituents. However, this technique is time-consuming, expensive, and often leads to misinterpretationof results, once the interactions among parametersare not taken into account(Hii et al., 2012). An alternative to overcome this technical limitation is the use of statistical designs coupled to response surface methodology, which allows the study of several factors

influencing the responses by varying them simultaneously and carrying out a limited number of experiments(Ye et al., 2010; Carneiro et al., 2013).

The objective of this study was to optimize the α-amylase production by *Escherichia coli* pAC92 using extruded bean as nitrogen and carbon source. A 3-factor simplex-lattice design coupled to response surface methodology (RSM) was applied to evaluate the combined effects of medium components on biomass and enzyme production. The information gathered was used to develop a mathematical correlation in searching of the optimum conditions for the growth and α-amylase production by the strain.

II. MATERIAL AND METHODS

2.1. Bacterial strain and inoculum preparation

The bacterium *Escherichia coli*pCA92 was used in this study. The strain was stored at -80 ºC in 15% (v/v) glycerol. For inoculum preparation, a single colony was picked up from the Luria-Bertani agar (enriched with 100 mcg/mLampicillin) and subcultured in 250-mL Erlenmeyer flasks containing 50 mL of Luria-Bertani (LB) broth and incubated at 37 ºC for 24 h to obtain an initial cell concentration with optical density (600nm) ranging from 0.4 to 0.6. The inoculum that consisted of 10% (v/v) of culture was used in all experimental runs.

2.2. Flour bean preparation and extrusion

The hardened beans (*Phaseolus vulgaris*and *Vigna unguiculata*) utilized for the extrusion process were provided by EMBRAPA Arroz e Feijão, Santo Antônio de Goiás, Goiás, Brazil. The grains were grounded in a Tecnal mill-grinder, sifted in a screen 0.42 mm and then extruded according to methodology described by Batista et al. (2010a). The extrudates were ground, sealed in plastic bags and refrigerated at 4 ºC until use.

2.3. Experimental design for medium formulation

A 3-factor simplex-lattice design with axial points and overall centroid was used to evaluate the effect of substitution of peptone and yeast extract by extruded flours from common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*). The mixture design allows studying the relationship between the proportion of the different nutrient sources and their respective responses in the optical density and α-amylase production by *Escherichia coli*pAC92. The design was implemented using Statistica 7.0 software (StatSoft Inc. Tulsa, OK, USA) and the factors defined as independent variables were peptone, yeast extract and extruded bean (Table 1).

2.4. Growth profile of *Escherichia coli*pAC92

The growth kinetic of *E.coli* pAC92 in the experimental media was compared with its growth in a commercial LB broth. For this, 5 mL of inoculumprepared in LB mediumwere centrifuged at 5000 g for 10 min. The medium-free cells were transferred to 0.15 mol/L sterile saline solution for reaching an absorbance of 0.5 at 600nm. Onemilliliter of cell suspension was transferred to 50 mL of the different media, incubated at 37 °C for 24 h under shaking (80 rpm). Aliquots of 1 mL were withdrawn every 2 h for determination of optical density and α-amylase activity. Collected aliquots were centrifuged at 5000 g for 10 min and the cell mass was re-suspended in 1 mL of 0.15 mol/L saline solution. The absorbance was determined at 600 nm by using a UV–VIS Spectrophotometer.

2.5. Determination of α-amylase activity

The enzymatic activity was evaluated from aliquots withdrawn during the growth kinetic experiment. The collected aliquots were centrifuged at 5000 g for 10 min and the cellular mass was washed twice with 0.15 mol/Lsaline solution. Then, the bacterial cellular wall was broken by using a saturated sucrose solution. The α-amylase activity was determined according to Bernfeld (1955), and the content of produced reducing sugar was determined following the methodology described by Miller (1959), using glucose as standard. One unit of enzyme was determined as the content in micrograms of reducing sugar released per milliliter of sample per minute of reaction.

2.6. Statistical analysis

The statistical analysis of the experimental mixture design was performed by multivariate analysis. The model was simplified to exclude terms that were not considered statistically significant ($p>0.05$) by analysis of variance (ANOVA). The quality of the polynomial model equation was judged by using the coefficient of determination adj-r^2. The mixture design and all subsequent tests were conducted in triplicate and the level of significance was 95%. All analyses were carried out using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

III. RESULTS AND DISCUSSION

3.1. Model establishment

The composition of a culture medium is one of the most important parameters to be analyzed in biotechnological processes with industrial purposes, because around 30-40% of the production costs were estimated to be accounted for the cost of the growth medium (Hajii et al., 2008; Batista et al., 2013). In addition, the cellular growth and protein expression by different microorganisms are greatly influenced by the media components, especially carbon and nitrogen sources(Ye et al., 2010; Rughoonundun et al., 2012).

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In this scenario, to study the feasibility of using extruded bean flours as a low cost nutrient source in substitution of peptone and yeast extract for growth of *E.coli*pAC92, a 3-factor simplex-lattice mixture design was used. The results of optical density of *E.coli*pAC92 using *Phaseolus vulgaris* and *Vigna unguiculata* as media constituents are shown in Table 1. As can be observed, the culture medium containing extruded common bean (*Phaseolus vulgaris*) presented values of optical density higher than those observed for cowpea (*Vigna unguiculata*).

Regarding to the effect of extruded common bean inclusion on the *E.coli* growth, the results from multivariate analysis evidenced that only the effect of interaction between the proportion of peptone and yeast extract (X_1X_2) did not affect the optical density of *E.coli*pAC92. On the other hand, the variable that most affected the response was the content of extruded bean (X_3), being observed a strong positive correlation with the optical density (r=0.93). The regression analysis showed an adequate fit of experimental values to the first-order polynomial model as a function of significant factors (adj-r^2=0.958). The mathematical model is represented in following equation:

$$OD_{600}(Phaseolus) = 0.20X_1 + 0.43X_2 + 1.35X_3 + 1.34X_1X_3 + 1.42X_2X_3 - 13.65X_1X_2X_3 - 1.84X_1X_2(X_1 - X_2) + 3.65X_1X_3(X_1 - X_3)$$

where X_1, X_2 and X_3 denotes peptone, yeast extract and extruded bean, respectively. The value of adjusted r^2 indicates that the proposed experimental model can determine 95.8% of the response variability.

Results of the multivariate analysis for the media using cowpea as nitrogen source evidenced that although the interaction between peptone and extruded bean (X_1X_3) had no effect on the response, all other variables significantly affected the growth of *E.coli*pAC92. In addition, the variable that most influenced the response was the content of extruded cowpea, which presented a positive correlation with the bacterial growth(r=0.78).

Using regression analysis, the polynomial equation that describes the correlation between the response and the significant variables can be represented by the following equation (adj-r^2=0.979):

$$OD_{600}(Vigna) = 0.19X_1 + 0.41X_2 + 0.74X_3 - 0.39X_1X_2 - 0.65X_2X_3 + 5.43X_1X_2X_3 - 3.69X_1X_2(X_1 - X_2) + 7.86X_1X_3(X_1 - X_3)$$

where X_1, X_2 and X_3 denotes peptone, yeast extract and extruded bean, respectively. The fitness of the model was expressed by the adj-r^2 value, which indicates that 97.9% of the variability in the response can be explained by the model. This suggested that the model accurately represents the data in the experimental region.

3.2. Response surface analysis

Multivariate design of experimentscoupled to response surface methodologyis a widely used method in optimization processes, once it consumes less time, requires a smaller set of experimental procedures and resources, allows the obtainment of large quantities of data in a single step process and enables the discoverof the most desirable conditions, or desirability(Yin et al., 2009; Mohamad et al., 2011; Boumba et al., 2013; Cheng et al., 2013).Hence, a response surface methodology (RSM) was used to determine the influence of nutrient sources (peptone, yeast extract and both extruded bean) in the *E.coli* growth.

Despite the good results of optical density in the media containing common bean and yeast extract or peptone in equivalent proportions, the surface response confirms the highest growth profile using extruded common beanas sole nitrogen source(Figure 1a).The projection evidenced a negative effect of peptone (run 1) and yeast extract (run 2) as isolated sources, while common bean shows a trend to a further growth by increasing its concentration.In this scenario, the mathematical prediction of an optimum composition is presented as 0.25% yeast extract and 0.75% of extruded bean. With this formulation, is expected to achieve an optimal density of 1.385 at 600 nm, a value very close to that previously observed in the culture medium containing extruded bean as unique nitrogen source (run 3).

This fact can be explained in terms of the nutritional requirements of the bacteria under study,which needs beyond the protein from peptone, also minerals and vitamins present in yeast extract. Thus, the improvement is probably due toa better nutritional balance betweenthe extruded bean and the yeast extract, encompassing not only a more complete source of nitrogen, but also essential vitamins and active compounds(Watson, 1976; Pepler, 1982; Batista et al., 2013).

The effect of inter-relations and interactions of the proportion of peptone, yeast extract and extruded cowpea on *E.coli*growth are depicted in Figure 1b. The response

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surface plot describes the variation on the response as a function of mixture composition. Unlike the extruded common bean, contradictory results were obtained from the medium containing extruded cowpea. The bacteria presented a greatest predicted value for optical density at the medium containing 0.75% peptone, 0.05% yeast extract and 0.20% of cowpea extruded, with values of OD_{600} of 0.897. However, in the tested compositions, results evidenced that the media containing extruded cowpea as unique nitrogen source (run 3) presented the highest optical density. In addition, the presence of extruded cowpea in low quantities positively affected the media containing peptone (run 7) and yeast extract (run 8) as main nitrogen source.

As can be observed in the surface plots depicted in Figure 1, there is a different influence of each bean specie over the *E.coli* growth, once the values ofoptical densitywere significantly different in each medium containing extruded bean. These results may be explained by the different intrinsic composition of each bean specie, especially regarding to the content of proteins and amino acid composition, as well as polysaccharide quantity and quality (Batista et al., 2010a,b). In this sense, the extrusion process interfered differently in each studied bean, modifying the biochemical proprieties and anti-nutritional components singularly.

Also, the lower values of optical density using cowpea as nutrient source can be due to the fact that cowpea presents storage proteins (vicilins) with biocidal activity(Ribeiro et al., 2007). It is possible that the extrusion process was not effective to inactivate these proteins, contributing to the lower efficiency of cowpea as nutrient source.

3.3. Growth profile of *Escherichia coli*AC92

Due to the constant modification in microbial genetics, new recombinant species and strains are frequently discovered and created. This demand requires new media formulation and optimization in industrial scale, continuously. However, this is a delicate process, since culture media formulation involves several variables and a large flow of data, which increase the difficult, especially when more than one variable changes at a time(Ye et al., 2010; Cofré et al., 2012; Delabona et al., 2013).In addition, an optimized medium has to be more profitable and efficient than traditional media. Thus, the growth profile of *E. coli* on media with higher optical density was compared to the growth profile in Luria-Bertanimedium (Figure 2).

As can be seen in Figure 2a, there was a clear improvement over the cellular growth in media containing extruded common bean as exclusive nutrient source (run 3), being observed an increase of 68% in the growth when extruded common bean was used as sole nutrient source and 20% when mixed with yeast extract (run 6).

Previous studies showed that LB medium contains large quantities of all of the essential inorganic compounds, necessary for the *E.coli*pAC92culture. Nevertheless, the carbon source is a probable limitation to a further growth(Sezonov et al., 2007). Considering that during the extrusion process of common bean, extremely high temperatures and pressure are reached, the modification of some polypeptide chains and the higher interaction between the molecules during the process, enhance the bioavailability of essential nutrients, such as carbon and nitrogen. This may be the reason for the improved efficiency of extruded common bean flouras *E.coli* pAC92 growth medium and acorroborationfor the successfully replacement of LB medium by this medium.

Despite the similar growth profile between cowpea bean medium and the commercial medium, the bacteria reached the saturation at an OD 18% inferior than LB medium, when cowpea was used as unique nitrogen source (Figure 2b). This may be due to a smaller amount of essential inorganic molecules available in the extruded cowpea, which causes a faster depletion of nitrogen and carbon, reaching the saturation earlier and limiting a further growth.

Once the culture medium containing extruded common bean was more effective than extruded cowpea for *E.coli* growth, the optimization tests for production of α-amylase were performed using extruded common bean as medium constituent.

3.4. α-amilase expression

It is known that high values of growth profile do not necessarily mean a proportional protein expression. Despite the culture medium containing extruded common bean had increased the growth of *E. coli*, changes in medium composition can interfere with the protein expression profile(Potvin et al., 2012; Carneiro et al., 2013). Aiming to verify the effectiveness of α-amylase production by *E.coli*pAC92in different composition media, tests were performed and the results are demonstrated in Table 2.

The results of the multivariate analysis evidenced that the effect of ternary interaction $X_1X_2X_3$ and the effects of binary interaction between the proportion of peptone and yeast extract (X_1X_2) and yeast extract and extruded bean (X_2X_3), did not affected significantly the production of α-amylase by *E. coli*(Figure 3a). In addition, the binary interaction between peptone and extruded common bean (X_1X_3) had the most pronounced effect on the response, presenting a strongly positive correlation with the α-amylase activity(r=0.92).

The regression analysis showed an adequate fit of experimental data to the full-cubic model as a function of significant variable. Thus, the polynomial equation that describes the correlation between the optical density and

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the media constituents is represented below (adj-r^2=0.993):

$$\alpha\text{-}amilase activity(U) = 2.16X_1 + 4.17X_2 + 3.58X_3 + 165.06X_1X_3 + 45.23X_1X_2(X_1 - X_2) - 318.27X_1X_3(X_1 - X_3)$$

This equation is based on the production of α-amylase as a function of the three different variables, concentration of peptone (X_1), concentration of yeast extract (X_2) and concentration of extruded common bean (X_3).The fitness of the model was expressed by the adj-r^2 value, which indicates that 99.3% of the variability in the response can be explained by the model.

In order to obtain the best condition for protein expression, diagrams of response surface were designed, presenting the effects of inter-relations and interactions of the different nutrient sources on theα-amylase expressionby *E.coli*pAC92 in media containing extruded common bean (Figure 3b).

The surface plot forα-amylase productionpresented an overall convex curvilinear profile. The mathematical prediction described the optimal medium as containing 0.30% of peptone and 0.70% of extruded bean, with maximal enzyme activity of 64.38 U.The optical density results on the other hand, shows a better condition in the medium composed exclusively by extruded bean (Table 1, run 3). These results show two different compositions of optimal media: one for cultivation and accumulation of biomass and other for protein production. However, the production of an optimized culture medium requires a unique formulation, efficient for cellular growth and protein expression, once is impracticable for industrial scale to manufacture two different media with the same components.

In this sense, a desirability test was performed aiming to obtain the values of the experimental variables that maximize both responses.The desirability function approach is one of the most widely used methods in industry for the optimization of multiple response processes, simultaneously. Desirability consists of an optimization method by combining all variables into a single objective function, which represents the relationship of all responses being optimized(Jeong and Kim, 2009; Costa et al., 2011). Figure 4 shows the diagrams describing the variation on the desirable response as a function of the mixture composition. In order to establish the most desirable media formulation for *E.coli* culture, the two main parameters were analyzed, optical density and α-amylase activity.

Results evidenced that the function D would be maximized by using a culture medium containing 0.5% of peptoneand 0.5% of extruded bean (run 5). In this condition,the mathematically predicted values for optical density and α-amylase activity were 1.111 and 44.60 U, respectively. These results were very close to those obtained in the experiments from the mixture design (Tables 1 and 2). Despite this medium did not present the highest optical density in first place, the growth profile was not significantly lower than in run 3, which presented the highest values of optical density.In view of the importance of an efficient nutrient supply for α-amylase expression in an optimized *E.coli* culture medium, the decrease of optical density can be disregarded.

Through desirability test, it was possible to reach a maximal optimization of this medium, encompassing production of cell mass and α-amylase expression in a one-step process. Once the production cost of extruded beans is cheaper than the production processes of yeast extract and peptone, this medium has an enormous potential, being extremely attractive for industrial and biotechnological applications.

IV. CONCLUSION

The mixture design and response surface analysis were found useful in locating the optimum level of the most significant factors that contribute to the maximum growth and α-amylase production by *Eschericia coli* pAC92. This study evidenced that the extruded common bean may be successfully used as substitute of peptone and yeast extract in culture media. The use of extruded bean improved the growth of*Escherichia coli*pAC92 as well as occasioned a good production of α-amylase. Therefore, considering the lower costs of extruded bean as nitrogen source, this substrate is financially attractive substitute of peptone and/or yeast extract in culture media formulation for the expression of α-amylase by*E.coli*pAC92 in an industrial scale production.

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Table.1: Mixture design employed for optimization of E.coli PAC92 growth using extruded beans as nutrient source.

Run	Peptone (X_1)	Yeast Extract (X_2)	Extruded Bean (X_3)	Optical Density of *E.coli*	
				Common bean	Cowpea bean
1	1% (1.0)	0% (0.0)	0% (0.0)	0.190	0.190
2	0% (0.0)	1% (1.0)	0% (0.0)	0.414	0.414
3	0% (0.0)	0% (0.0)	1% (1.0)	1.333	0.746
4	0.5% (0.5)	0.5% (0.5)	0% (0.0)	0.205	0.205
5	0.5% (0.5)	0% (0.0)	0.5% (0.5)	1.082	0.496
6	0% (0.0)	0.5% (0.5)	0.5% (0.5)	1.215	0.420
7	0.66% (0.67)	0.17% (0.17)	0.17% (0.17)	0.521	0.589
8	0.17% (0.17)	0.66% (0.67)	0.17% (0.17)	0.641	0.612
9	0.17% (0.17)	0.17% (0.17)	0.66% (0.67)	0.934	0.178
10	0.33% (0.33)	0.33% (0.33)	0.34% (0.33)	0.294	0.561

Values in bracket correspond to the coded variable level.

Table.2: Mixture design matrix used for optimization of α-amylase production by E.coli pAC92 using extruded common bean as nutrient source.

Run	Peptone (X_1)	Yeast Extract (X_2)	Extruded Bean (X_3)	α-amylase activity (U)	
				Observed	Predicted
1	1% (1.0)	0% (0.0)	0% (0.0)	2.39	2.16
2	0% (0.0)	1% (1.0)	0% (0.0)	4.39	4.17
3	0% (0.0)	0% (0.0)	1% (1.0)	3.81	3.58
4	0.5% (0.5)	0.5% (0.5)	0% (0.0)	2.99	2.53
5	0.5% (0.5)	0% (0.0)	0.5% (0.5)	44.59	44.13
6	0% (0.0)	0.5% (0.5)	0.5% (0.5)	3.83	3.37
7	0.66% (0.67)	0.17% (0.17)	0.17% (0.17)	5.04	6.41
8	0.17% (0.17)	0.66% (0.67)	0.17% (0.17)	4.77	6.14
9	0.17% (0.17)	0.17% (0.17)	0.66% (0.67)	38.64	40.01
10	0.33% (0.33)	0.33% (0.33)	0.34% (0.33)	24.87	22.82

Values in bracket correspond to the coded variable level.

Figure captions

Figure 1. Mixture contour maps showing the effect of three variables on the optical density of *E.coli* pCA92. (a) yeast extract, peptone and extruded common bean or (b) yeast extract, peptone and extruded cowpea.

Figure 2. Effect of different nitrogen sources in the growth of *Escherichia coli*: (a) mixture design using common bean as nitrogen source; (b) mixture design using extruded cowpea as nitrogen source. As the control, the microorganism was grown in a commercial Luria-Bertani medium. Results are means ± standard deviation of triplicate samples.

Figure 3. Pareto chart (a) and mixture contour map (b) for the α-amylase activity in the mixture design experiments. In the Pareto chart, the horizontal bar represents the ratio between the effects of variables and their respective standard error. All tests were conducted in triplicate.

Figure 4. Response surface plot of desirability as a function of the proportion of yeast extract, peptone and extruded common bean.

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Figure 1

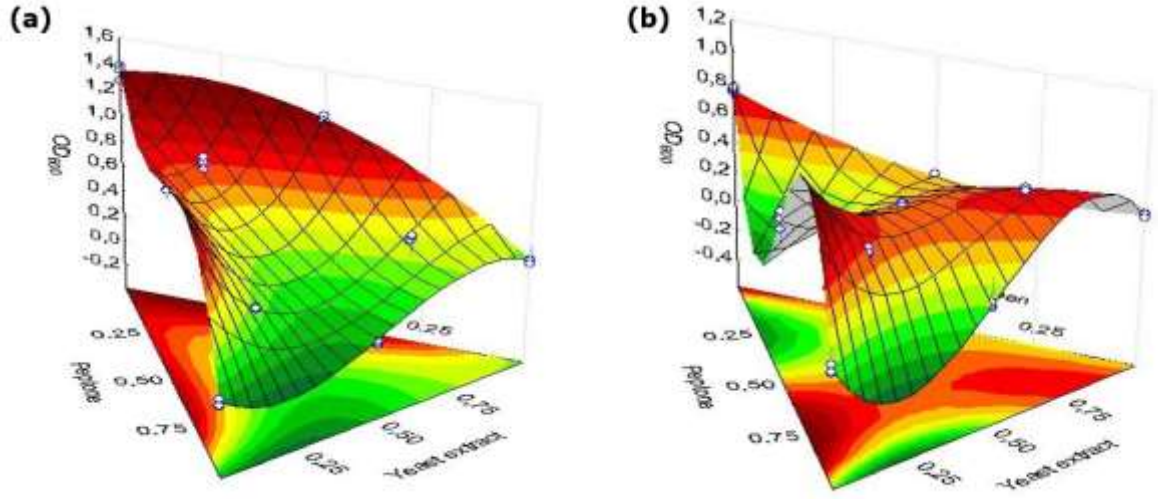

Figure 2

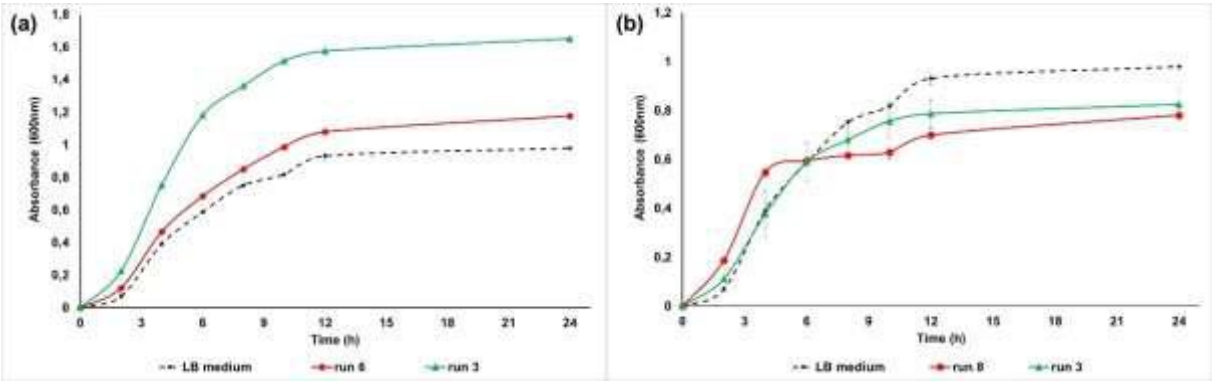

Figure 3

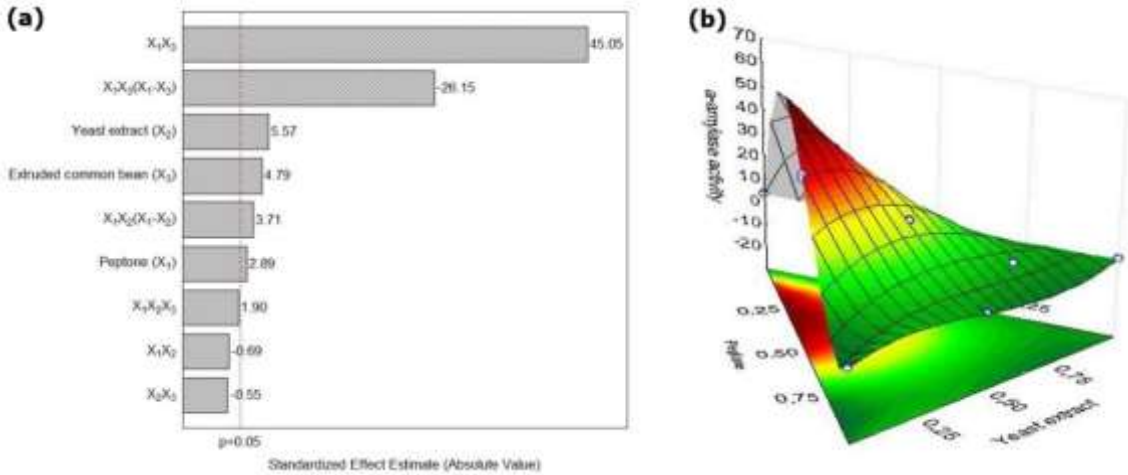

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Figure 4