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Production of transglutaminase by Streptoverticillium ladakanum using sodium caseinate, glycerol and noncrystallized carbohydrates from sugar cane molasses

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Abstract. Transglutaminase is an enzyme that catalyses an acyl transfer reaction between γ-carboxamide groups of glutaminyl residues and ε-amino grups of lysine residues in proteins. Due to this property, this enzyme is used for enhancing textural properties of protein-rich food. The transglutaminase used as food additive is obtained by microorganisms, mainly by *Streptoverticillium ladakanum*. For the production of microbial transglutaminase, nitrogen and carbon sources are needed. In this work, the effect of sodium caseinate concentration (ranging from 20 to 60 g/L); glycerol concentration (ranging from 20 to 60 g/L) and the concentration (ranging from 30 to 60 g/L) of total noncrystallized carbohydrates (saccharose, glucose, and fructose) from sugar cane molasses on the enzyme activity using *Streptoverticillium ladakanum* NRRL 3191 was tested. Enzyme activity was measured at given incubation times (48, 72, 96 and 120h) in a batch system at 400 rpm. Data were fitted with a Box Behnken model using the Minitab®15 Statistical Sofware from Minitab Inc. Results suggest that sodium caseinate, glycerol and carbohydrates from sugar cane molasses can be used for biotechnological production of transglutaminase due to the fact that the enzyme activity was increased by lowering the levels of studied factors, showing that the combination of sodium caseinate, 20 g/L; glycerol, 20 g/L, and total noncrystallized carbohydrates from sugar cane molasses 30 g/L yielded the higher value of transglutaminase produced, 0.4 U/mL at 96 and 120 h. However, in order to optimize the process, it is necessary to carry out further experiments.

Keywords. Microbial transglutaminase, food additive, sodium caseinate, glycerol, sugar cane molasses

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Introduction

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase: E.C. 2. 3. 2. 13) catalyses an acyl-transfer reaction between a γ-carboxyamide group of glutamine (Gln) residues (acyl donors) and primary amines (acyl acceptors) from an ε-amino group of a Lys residue in proteins (Yeo et al., 2009). Transglutaminases are enzymes widely distributed in living organisms such as archaea, bacteria and eukariotic cells, and their properties have been extensively studied (Makarova et al., 1999). All kinds of transglutaminase are similar with each other. Transglutaminases from animal or vegetal tissues are Ca^{+2} dependant and difficult to extract from the natural source. However, bacterial transglutaminases do not require Ca^{+2} to catalize the chemical bonding reactions (Griffin, et al., 2002).

The interest on the applicatinos of transglutaminase for the food industriy is remarkable due to the cappability of this enzyme to catalyze cross linking reactions in protein – rich food products. The application of trasglutaminase stabilise milk yoghurt improving properties without undesired structural changes during storage (Guyot and Kulozic, 2011), and can improve the emulsifying properties of milk avoiding coalescence through cross-linking of milk proteins. Ramirez et al. (2007) reported the application of transglutaminase to obtain low salt restructured fish products. Additionally, recent advances on the application of transglutaminase for the production of biopolymers from whey protein and gelatin from collagen have been reported (Hernàndez – Balada et al., 2009), and for the production of bovine serum albumin gels (Gan et al., 2009).

On the other hand, studies on the microbial producion of transglutaminase have also been reported. For examlple Yeo et al. (2009) reported a new *Streptomyces* strain from a soil sample, characterise the microorganism and propose the optimal culture media for the microbial production of a transglutaminase: It was found for this enzime not to require Ca^{+2} for catalysis. The production of microbial transglutaminase on media made from sorgum straw hydrolizates has also been reported (Téllez-Luis et al. 2004a). Noda et al. (2012) studied the production of translgutaminase using a recombinant *Streptomyces lividans cultures* on glycerol, starch, xylo-oligosacharides and cellobiose, the results are promising due to the hability for the microorganism to use a variety of carbon sources to produce the enzyme. Besides, the effect of the heat shock, methanol and salt stress on the enhancement of transglutaminase production in Streptomyces mobaraensis DSM 40587 was recently studied. The results showed that stress caused by salt concentration was the most effective among the stress sources studied, resulting in a 3.5 – fold increased the production of transglutaminase (Zhang et al. 2012).

Regarding the biotechnological production of microbial transglutaminase, it has been reported that the culture media can represent almost 30% of the total cost for the production above all when using high cost sustrates like glycerol (Portilla et al., 2009). Glycerol has been reported to be a good sustrate for biotechnological aims on the production of a variety of compounds including transglutaminase (Téllez-Luis et al., 2004b; da Silva et al., 2009), however it is necesary to define low cost sustrates like sugar cane molasses. The use of sugar cane molasses has been extensively studied for biotechnological aims (Ravi Kumar et al., 2010; Maiti et al., 2011). Furthermore, the feasibility of using mixtures of glycerol/sugar cane molasses for the production of transglutaminase was previously reported (Portilla et al. 2009), but research related to the effect of differents concentration of nutrients on the production of transglutaminase is needed. In addition, the effect of casein on both, the production of transglutaminase and the growth of the *Streptoverticillium cinnamoneum* was proved to be significant (Junqua et al., 1997), but no strategies for optimization of the amount of casein used

were stablished. In this work, the effect of sodium caseinate concentration (ranging from 20 to 60 g/L); glycerol concentration (ranging from 20 to 60 g/L) and the concentration (ranging from 30 to 60 g/L) of total noncrystallized carbohydrates (saccharose, glucose, and fructose) from sugar cane molasses on the enzyme activity using *Streptoverticillium ladakanum* NRRL 3191 was tested.

Safety Emphasis

In this study safety was extremely important, as it consisted in handling microorganisms. In fact *Streptoverticillium* strains for transglutaminase production are known to be not harmful for humans. The reason for considering safety this study is the integrity of the strain used, as it can be invaded by native bacteria in sugar cane molasses or bacteria from the environment that can find an oportunity to grow on the media for transglutaminase production. A higly strict procedure for sterilization is necessary for both aims, to eliminate all invading bacteria from raw materials, and to avoid damage of the sugars contained in sugar cane molasses solutions. Sterilization of sugar cane molasses solutions by filtration separately from the other nutrients is necessary. Seterilization by heating is necessary for other nutrients. The second topic on safety considered in this work, is related to the purity of the transglutaminase solutions obtained. Transglutaminase is intented to be used for food products, therefore it is neccessary to be absolutely free of bacterial cells. Due to the fact that transglutaminase is extracellular, a sedimentation followed by a filtration procedure is applied to ensure the absence of cells that can affect the quality of the tarjet product for transglutaminase applications. In this terms, to improve the safety it is neccessary to work in an aseptic environment, and to take control of the parameters for steriliztion by heating. Furthermore, a strict separation proccess and further purification of the crude extracts need to be applied for ensuring safety of the enzyme produced.

Materials and methods

Carbon and nitrogen sources and culture media

Sugar cane molasses was kindly supplied by a local sugarmill. Glycerol and sodium caseinate was purchased from Sigma Aldrich. In order to find the conditions for culture media preparation, preliminary growth kinetics were performed. The conditions found in the preliminary study were: sterilzation of nutrients separately; sugar cane molasses solition containing the stablished noncrystallized carbohydrates concentration was sterilized by filtration, and all other nutrients including glycerol and sodium caseinate in the stablished concentrations were sterilized by autoclave at 120 ºC during 15 min.

Microorganism and inoculum preparation

The microorganism used in this study was *Streptoverticillium ladakanum* NRRL – 3191, procured from the Agricultural Research Services (ARS) cuture collection, IL, USA. The lyophilized was cultured in a broth containing yeast extract (4 g/L), malt extract (10 g/L), glucose (5 g/L) and water (100 mL) for over 48 h. An alicuot of 5 mL was transferred to a broth containing sodium phosphate dibasic (5 g/L); potasium phosphate monobasic (2 g/L); magnesium sulfate heptahydrate (0.5 g/L); peptone (10.5 g/L); yeast extract (2.5 g/L); sodium caseinate (38.4 g/L); glycerol (30 g/L); water (100 mL). After 96 h, alicuots of 1 mL were mixed with 1mL of glycerol and kept under -60 ºC until use. A sample of the culture was taken and cultured for 48 h on the same composition media. An aliquot (5mL) of this culture was used as inoculum for the study.

Trangslutaminase activity measurements

In order to follow the enzyme production on the culture media, the activity of the ezyme was measured by the colorimetric method based on the formation of hydroxamate from *N*-carbobenzoxy-L-glutaminylglycine (Grossowicz et al. 1950). One unit of enzyme activity (U) was defined as the as the amunt that causes the formation of 1µMol de monohydroxamate in 1 min at 37ºC. Reagents for transglutaminase activity measurements were purchased from Sigma Aldrich.

Experimental design and statistical analysis

In order to test the effect of sodium caseinate concentration, glycerol concentration, and the concentration of total noncrystallized carbohydrates from sugar cane molasses on the enzyme activity using *Streptoverticillium ladakanum* NRRL 3191, a Box Behneken model was stablished under the conditions given in table 1. Experimental data were analyzed by the Response Surface Methodology using the Minitab®15 Statistical Sofware from Minitab Inc. Where the interrelationship between dependent and operational variables was established by a model including linear, interaction and quadratic terms with α 0.05, according to the equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

where Y is the dependent variable, β denotes the regression coefficients, and X denotes the independent variables.

Table 1. Variables used in the study.

Fixed variables	**Value**	**Units**	
Temperature	26 °C	^{o}C	
pH	7.2	-	
Agitation speed	400	RPM	
Independent variables	**Nomenclature**	**Units**	**Range**
Sodium caseinate	SC	g/L	20-60
Glycerol	G	g/L	20-60
Total noncrystallized carbohydrates from sugar cane molasses (Glucose, fructose and sucrose)	TNC	g/L	30-60
Normalized independent variables	**Nomenclature**	**Definition**	**Range**
Sodium caseinate	X_1	(SC-40)/20	-1 to 1
Glycerol	X_2	(G-40)/20	-1 to 1
Total noncrystallized carbohydrates from sugar cane molasses (Glucose, fructose and sucrose)	X_3	(TNC-45)/15	-1 to 1
Dependent variables	**Nomenclature**	**Units**	
Enzyme activity at 72 h	E72h	U/mL	
Enzyme activity at 96 h	E96	U/mL	
Enzyme activity at 120 h	E120	U/mL	

Results and discussions

Table 2 shows the results obtained from the activity measurements at given times. Table 3 shows the parameters for models obtained at given times. Regarding the model for E72h it can be seen that G and TNC are the active effects in the model, however, it is not possible to consider these effects due to the fact that this model can barely fit to the results obtained as the R^2 value is 89.55%. Regarding the model for E96h, it is noticeable that the three factos are active as the p values are lower than alpha, wich is statistically significative. Additionally, the effects can be considered for further predictions as the R^2 with value of 94.22 fairly fit the data. The same statistical behaviour is observed for the model E120h. It is also noticeable from the information obtained that no interaction between variables or second order interactions were observed, which is in agreement to the previous findings where glycerol and sugar cane molasses were used as carbon sources (Portilla et al., 2009). Regarding to interactions of factors with SC, the results obtained in this work agree with previous conclusions about the role of casein as inducer for transglutaminase production (Junqua et al., 1997).

Table 2. Results obtained for the enzyme activity at given times.

Experiment number	Independent variables			Dependent variables		
	X_1	X_2	X_3	E72h	E96h	E120h
1	0	-1	-1	0.16	0.19	0.22
2	-1	-1	0	0.21	0.37	0.39
3	1	-1	0	0.10	0.12	0.16
4	0	-1	1	0.02	0.11	0.17
5	-1	0	-1	0.10	0.26	0.26
6	1	0	-1	0.09	0.09	0.11
7	0	0	0	0.01	0.10	0.14
8	0	0	0	0.06	0.10	0.16
9	0	0	0	0.06	0.12	0.17
10	-1	0	1	0.00	0.10	0.10
11	1	0	1	0.00	0.08	0.01
12	0	1	-1	0.06	0.12	0.14
13	-1	1	0	0.03	0.10	0.12
14	1	1	0	0.02	0.03	0.03
15	0	1	1	0.00	0.00	0.00

Table 3. Parameters for models obtained at given times.

	E72h		E96h		E120h	
	Coeficient	p value	Coeficient	p value	Coeficient	p value
Constant	0.043333	0.076	0.10667	0.004	0.156667	0.000
X_1	-0.01625	0.23	-0.06375	0.004	-0.07	0.001
X_2	-0.0475	0.01	-0.0675	0.003	-0.08125	0.001
X_3	-0.04875	0.009	-0.04625	0.015	-0.05625	0.003
X_1^2	0.017083	0.373	0.03792	0.100	0.002917	0.862
X_2^2	0.029583	0.151	0.01042	0.604	0.015417	0.378
$\mathbf{X_3^2}$	-0.012917	0.493	-0.01208	0.549	-0.039583	0.056
$\mathbf{X_1X_2}$	0.025	0.197	0.045	0.055	0.035	0.071
$\mathbf{X_1X_3}$	0.0025	0.887	0.0375	0.093	0.015	0.372
X_2X_3	0.02	0.287	-0.01	0.604	-0.0225	0.202
R^2	89.55		94.22		96.57	

Regarding to the analysis of the effect of factors on the production of transglutaminase, it is noticeable that the enzyme activity was increased by lowering the levels of the three factors, this result is showed in figure 1 which is related to the E96h model with the TNC level fixed at the lower value (30 g/L). Besides, it is remarkable that the effect of SC is higher than the effect of G. The lower the amount of SC the higuer enzime activity. This effect can explained the by the crosslinking of the sodium caseinate molecules.

On the orher hand, the low effect of glycerol can be of great importance due to the fact that it is more expensive than sugar cane molasses as carbon sources, therefore the cost of the media can be lowered as lower amounts of this carbon source can be used for the production of transglutaminase. The results show that the combination of SC, 20 g/L; G, 20 g/L, and TNC 30 g/L can yield the higher value of transglutaminase as it is predicted for the model, which is up tp 0.4 U/mL. These results are similar to those corresponding to the E120h model.

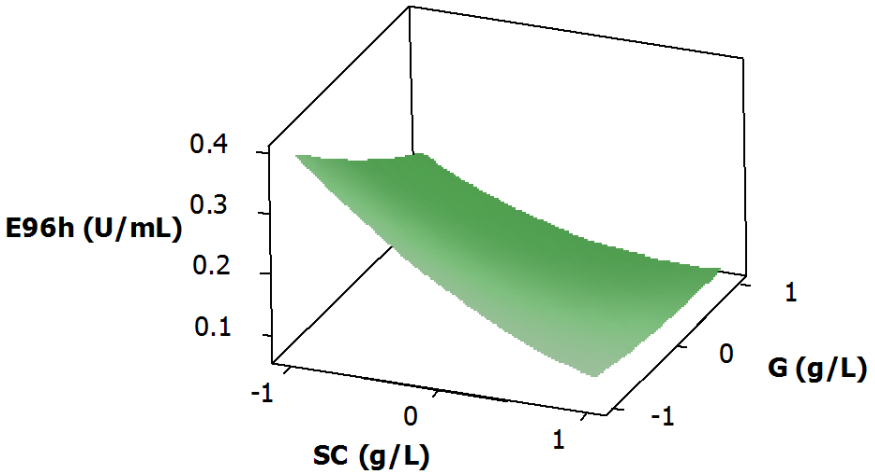

Figure 1. Surface plot of E96h showing the dependence of the enzyme activity from the concentrations (g/L) of SC and G, fixing the value for the TNC at the lower level (30 g/L).

It is also interesting to remark the effect of TNC on the enzyme activity, which is showed in Figure 2. It is noticeable that by increasing the concentration of TNC a lower enzyme activity is obtained. This culd be due to the high solute concentration in media.

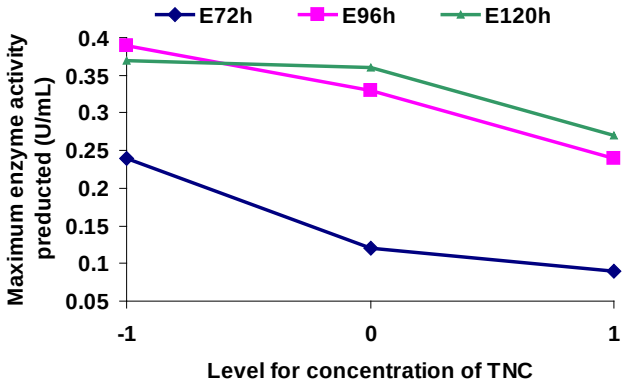

Figure 2. Effect of TNC on the maximum enzyme activity predicted for the three models.

The transglutaminase production was found in this study is low in comparison with other results previously reported (Téllez – Luis et al. 2004b and Zhang et al. 2012) using conventional carbon sources. However the results obtained in this study are higher in comparison whith the production of transglutaminase using other non conventional carbon sources like sorgum straw hydrolysates (Téllez – Luis et al., 2004a).

Conclusion

The use of sodium caseinate, glycerol and sugar cane molasses is suitable for the production of transglutaminase. The effect of these nutrients in the process is statistically significant. No interaction effect or a second order effect of the parameters is noticeable in the range studied. The lowest concentrations of these nutrientes lead to the higher production of translgutaminase as it is shown by the enzyme activity measured. However, in order to enhance and to optimise the production of transglutaminase using sodium caseinate, glycerol and sugar cane molasses as nutrients, further work has to be done.

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