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Optimization of Composition of Media for the Production of Extracellular Glucoamylase by *Candida guilliermendii*

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Abstract: The combined effects of macronutrients of media on glucoamylase production by *Candida guilliermendii* were studied using Design Of Experiment (DOE). A 2^{P-k} factorial design was chosen to explain fifteen medium constituents: pH, Starch, Sucrose, Yeast extract, Peptone, NH_4Cl $(NH_4)_2HPO_4$, NH_4NO_3, CH_4NO_2 $(NH_4)_2SO_4$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$ and $MgCl_2$ and analyse the results. This procedure limited the number of actual experiments performed while allowing for possible interactions between components. The p-value of the coefficient for quadratic effect of pH, starch and yeast extract concentration was <0.001, suggesting that they were the main experimental variables having the highest effect on the production of glucoamylase. It was found that yeast extract had a great effect on glucoamylase production. The optimal combinations of media constituents for maximum which were chosen for further studies on production of glucoamylase were determined as 10 g L^{-1} starch, 0.45 g L^{-1} urea, 0.61 g L^{-1} NH_4NO_3, 3 g L^{-1} Yeast extract and 0.1 g L^{-1} Mg SO_4.

Key words: *Candida guilliermendii*, glucoamylase, fermentation, Design Of Experiment (DOE)

INTRODUCTION

Amylases are among the most important enzymes used in biotechnology, particularly in processes involving starch hydrolysis. Though amylases originate from different sources (plants, animals and micro organisms), the microbial amylases are the most used in industry, due to their reproductivity (Burhan *et al*., 2003). Natural fermented media (foods, soils and waste) offer sources for isolation of micro-organism strains producing amylases. Many strains used in food industry are isolated from fermented food media (Pandey *et al*., 2000; Burhan *et al*., 2003; Gomes *et al*., 2003).

Various industries, such as food, brewing, textile, pharmacy and confectionaries depend on the various products especially extra-cellular enzymes produced by micro-organisms (Grupta *et al*., 2003). An extra-cellular amylase, specifically raw starch digesting amylase has found important applications in bioconversion of starches and starch-based substrates (Forgarty, 1983; Okolo *et al*., 1995).

Optimization of medium by the classical method involves changing one independent variable keeping the other factors constant. The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors (Liu and Tzeng, 1998). In fermentation process, the operational variables interact and influence each other. As a result, it is important that the optimization method accounts for the interactions so that a set of optimal experimental condition can be determined (Silva and Roberto, 2001). This limitation of a single factor optimization process can be eliminated by different techniques.

The need of efficient methods for screening large number of variables has led us to the adoption of statistical experimental designs. Statistical methods of Placket-Burman (1946) used in this work have been applied to bacterial culture optimization (Ahuja *et al*., 2004) and animal cell culture (Ganne and Mignot, 1991).

Such statistical design have already been used in many research works; such as the optimization of amylase and protease production from *Aspergillus awamori* (Negi and Banerjee, 2006) and the optimization of α-amylase production by Aspergillus niger (Djekrif-Dakhmouche *et al*., 2006), *Aspergillus oryzae* (Bennamoun *et al*., 2004; Francis *et al*., 2003) and by *Bacillus* sp. (Saban Tanyildizi *et al*., 2005). These designs

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were also used for the selection of amino acids causing the increase of the production of pyoverdine by *Pseudomonas fluorescens* (Kissalita *et al*., 1993) and the optimization of the production of carotenoids by *Rhodotorula glutinis* DBVPG 3853 (Buzzini, 2000).

The present study is aimed to determine better conditions for growth and the glucoamylase productivity, particularly their behaviour toward pH and compositions of media using statistical design. The choice of yeast isolation was justified by the facility of their culture and their harmlessness.

MATERIALS AND METHODS

Microorganism used: *Candida guilliermendii* was isolated in Laboratory of Microbial Biotechnology (LMB) from traditional Moroccan sourdough using the following medium: soluble starch (5 g L^{-1}), KH_2PO_4 (3 g L^{-1}); $(NH_4)_2SO_4$ (1 g L^{-1}); $MgSO_4$ (0.5 g L^{-1}); yeast extract (4 g L^{-1}). pH was adjusted to 5 with HCl 0.1M. The medium was solidified by the addition of 1.5% agar and autoclaved at 121°C for 15 min. Liquid medium was incubated in flask on a rotary shaker set at 105 rpm for 72 h. Incubation was at 30°C.

Growth rate: Growth rate was determined by measuring the absorbance of the suspension at 600 nm.

Cultivation and production of glucoamylase by *Candida guilliermendii*

Enzyme assays: The fermented broth was taken after 72 h and centrifuged at 7000 rpm for 10 min and then substrate free supernatant was used for estimation of enzyme activity.

Glucoamylase activity was determined by measuring the reducing sugar formed by the enzymatic hydrolysis of starch using the method of Somogyi and Nelson (Nelson, 1944). 0.25 mL soluble starch (1%), 0.15 mL phosphate buffer (0.1M), 0.1 mL enzyme solution were mixed and incubated at 40°C in water bath for 30 min, the reaction was stopped by 2 mL of Somogiy reactive and 1.5 mL of distilled water, followed by boiling for 15 min to develop blue colour. The absorbance measured at 540 nm with a spectrophotometer. The activity was measured against the control in which no enzyme was added. A calibration curve of absorbance and concentration of glucose was established with known amount of glucose.

One unit (µmol/L/min) of amylase was defined as the amount of µmol of reducing sugar per litre of enzymes per min, measured as glucose under the conditions of assay.

Total protein concentration was measured by the method of Bradford. The samples were read at 595 nm against the blanks with the same compositions as the samples

Experimental design: The Plackett-Burman experimental design assumes that there are no interactions between the different media constituents, xi in the range of variables under consideration (Plackett and Burman, 1946). A linear approach is considered to be sufficient for screening.

$$Y = \beta 0 + \Sigma \beta i x i \ (I = 1, \ldots, k)$$

where, Y is the estimated target function and βi are the regression coefficients. The Plackett-Burman experimental design is a fractional factorial design and the main effect (the contrast coefficient) of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (-1)

Table 1: Summary of variables for the (Plakett-Burman) design for the optimization of parameters

Aim of the study	Study of the effects
No. of variables	15
No. of experiments	32
No. of coefficients	16
No. of answers	4

Table 2: Experimental range and levels of the independent variables

Factors		No. of levels	Levels
pH	b1	2	5.00
			7.00
Starch	b2	2	5.00
			10.00
Sucrose	b3	2	0.00
			2.00
NH_4Cl	b4	2	0.00
			0.81
$(NH_4)_2HPO_4$	b5	2	0.00
			1.00
NH_4NO_3	b6	2	0.00
			0.61
CH_4NO_2	b7	2	0.00
			0.45
$(NH_4)_2SO_4$	b8	2	0.00
			1.00
Yeast extract	b9	2	0.00
			3.00
Peptone	b10	2	0.00
			3.00
$CaCl_2$	b11	2	0.00
			0.10
$MnCl_2$	b12	2	0.00
			0.10
$FeCl_2$	b13	2	0.00
			0.10
$ZnCl_2$	b14	2	0.00
			0.10
$MgCl_2$	b15	2	0.00
			0.10

Table 3: Matrix of the experimental design using Plackett-Burman method for screening of nutrients

Exp.	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15
1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1
2	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1
3	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1
4	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1
5	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1
6	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1
7	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1
8	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1
9	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1
10	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1
11	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1
12	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1
13	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1
14	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1
15	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1
16	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1
17	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1
18	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1
19	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1
20	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1
21	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1
22	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1
23	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1
24	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1
25	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1
26	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1
27	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1
28	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1
29	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1
30	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1
31	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
32	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

The whole factors tested are represented in Table 1 and 2. The matrix used in this study is represented in Table 3, comprising 16 experiments and 15 factors. Each line represents the various experiments and each column represents the various factors. The last line is always taken on the level (-1). In order to determine non controlled residues and allow for the estimation of the experimental errors, each experiment was repeated twice (32 experiments).

RESULTS AND DISCUSSION

Experimental design (Table 4) was carried out according to the design for 72 h at 30°C, under agitation on rotary shaker at 105 rpm. The fermented samples were extracted and assayed for biomass, glucoamylase activity, protein and final pH. The results were analyzed by statistical software. The design and results of experiments carried out with the placket-Burman design are given in Table 5.

The analysis of variance (ANOVA) was calculated for each response for the determination of significant parameters. ANOVA consists of classifying and cross-classifying statistical results and testing whether the means of a specified classification differ significantly. This was carried out by Fisher's statistical test for the analysis of variance. The F-value standing for the ratio of the mean square due to regression upon the mean square due to error indicates the influence of each controlled factor on the tested model.

The effect of the studied factors on the production of biomass: The effect of the factors upon the biomass is reported in Table 6.

Values of Probability > F less than 0.05 (0.01) indicated that model terms were significant. In this response (biomass production) the model was found significant. The biomass production model determination coefficient R^2 (0.998) strongly suggested that the fitted model could explain 99.8% of the total variation. Noises slightly affect the model. This implies a satisfactory representation of the process by the model.

The variation in pH from 5 to 7 leads to a negative effect on the production of biomass (PP 99%). These results may be explained by the fact that adequate development of yeasts necessitates a rather acid pH, the optimal growth pH of which is 5 (Botton *et al*., 1990; Martinilli and Kinghorn, 1997).

Of the carbon sources tested, starch showed a great effect on the production of biomass (99%), while sucrose had no effect on this production, which leads us to conclude that starch constitutes an adequate carbon

Table 4: Experimental design (Plackett-Burman) used to optimize the parameters for the production of glucoamylase by *Candida guilliermendii*

Exp.	Rand	pH	Starch	Sucrose	NH_4Cl	$(NH_4)_2HPO_4$	NH_4NO_3	CH_4N_2O	$(NH_4)_2SO_4$	Yeast extract	Peptone	$CaCl_2$	$MnCl_2$	$FeCl_2$	$ZnCl_2$	$MgCl_2$
1	26	7	6	2	0.81	0	0.61	0.00	1	3	0	0.0	0.1	0.0	0.0	0.0
2	5	7	6	2	0.81	0	0.61	0.00	1	3	0	0.0	0.1	0.0	0.0	0.0
3	12	5	6	2	0.81	1	0.00	0.45	0	3	3	0.0	0.0	0.1	0.0	0.0
4	16	5	6	2	0.81	1	0.00	0.45	0	3	3	0.0	0.0	0.1	0.0	0.0
5	10	5	5	2	0.81	1	0.61	0.00	1	0	3	0.1	0.0	0.0	0.1	0.0
6	25	5	5	2	0.81	1	0.61	0.00	1	0	3	0.1	0.0	0.0	0.1	0.0
7	23	5	5	0	0.81	1	0.61	0.45	0	3	0	0.1	0.1	0.0	0.0	0.1
8	29	5	5	0	0.81	1	0.61	0.45	0	3	0	0.1	0.1	0.0	0.0	0.1
9	14	7	5	0	0.00	1	0.61	0.45	1	0	3	0.0	0.1	0.1	0.0	0.0
10	2	7	5	0	0.00	1	0.61	0.45	1	0	3	0.0	0.1	0.1	0.0	0.0
11	31	5	6	0	0.00	0	0.61	0.45	1	3	0	0.1	0.0	0.1	0.1	0.0
12	7	5	6	0	0.00	0	0.61	0.45	1	3	0	0.1	0.0	0.1	0.1	0.0
13	32	5	5	2	0.00	0	0.00	0.45	1	3	3	0.0	0.1	0.0	0.1	0.1
14	3	5	5	2	0.00	0	0.00	0.45	1	3	3	0.0	0.1	0.0	0.1	0.1
15	20	7	5	0	0.81	0	0.00	0.00	1	3	3	0.1	0.0	0.1	0.0	0.1
16	24	7	5	0	0.81	0	0.00	0.00	1	3	3	0.1	0.0	0.1	0.0	0.1
17	17	7	6	0	0.00	1	0.00	0.00	0	3	3	0.1	0.1	0.0	0.1	0.0
18	28	7	6	0	0.00	1	0.00	0.00	0	3	3	0.1	0.1	0.0	0.1	0.0
19	18	5	6	2	0.00	0	0.61	0.00	0	0	3	0.1	0.1	0.1	0.0	0.1
20	30	5	6	2	0.00	0	0.61	0.00	0	0	3	0.1	0.1	0.1	0.0	0.1
21	27	7	5	2	0.81	0	0.00	0.45	0	0	0	0.1	0.1	0.1	0.1	0.0
22	9	7	5	2	0.81	0	0.00	0.45	0	0	0	0.1	0.1	0.1	0.1	0.0
23	13	5	6	0	0.81	1	0.00	0.00	1	0	0	0.0	0.1	0.1	0.1	0.1
24	15	5	6	0	0.81	1	0.00	0.00	1	0	0	0.0	0.1	0.1	0.1	0.1
25	21	7	5	2	0.00	1	0.61	0.00	0	3	0	0.0	0.0	0.1	0.1	0.1
26	4	7	5	2	0.00	1	0.61	0.00	0	3	0	0.0	0.0	0.1	0.1	0.1
27	1	7	6	0	0.81	0	0.61	0.45	0	0	3	0.0	0.0	0.0	0.1	0.1
28	8	7	6	0	0.81	0	0.61	0.45	0	0	3	0.0	0.0	0.0	0.1	0.1
29	22	7	6	2	0.00	1	0.00	0.45	1	0	0	0.1	0.0	0.0	0.0	0.1
30	11	7	6	2	0.00	1	0.00	0.45	1	0	0	0.1	0.0	0.0	0.0	0.1
31	6	5	5	0	0.00	0	0.00	0.00	0	0	0	0.0	0.0	0.0	0.0	0.0
32	19	5	5	0	0.00	0	0.00	0.00	0	0	0	0.0	0.0	0.0	0.0	0.0

Table 5: Observed responses and calculated values

	Biomass (Do 6000)		Glucoamylase (μmol/min/L)		Proteins (mg L^{-1})		Final pH	
Exp.	Y exp.	Y calc.	Y exp.	Y calc.	Y exp.	Y calc.	Y exp.	Y calc.
1	6.776	6.8005	2483.77	2471.675	0.742	0.729	7.28	7.270
2	6.825	6.8005	2459.58	2471.675	0.715	0.729	7.26	7.270
3	8.946	8.9390	2609.55	2610.060	1.610	1.602	7.21	7.205
4	8.932	8.9390	2610.57	2610.060	1.594	1.602	7.20	7.205
5	3.470	3.5130	304.69	315.240	1.047	1.045	6.99	6.995
6	3.556	3.5130	325.79	315.240	1.043	1.045	7.00	6.995
7	6.265	6.1950	1445.10	1462.035	1.175	1.179	7.26	7.265
8	6.125	6.1950	1478.97	1462.035	1.183	1.179	7.27	7.265
9	1.615	1.6400	1487.03	1508.000	0.564	0.570	7.13	7.130
10	1.665	1.6400	1528.97	1508.000	0.575	0.569	7.13	7.130
11	8.113	8.1470	1737.03	1745.900	1.777	1.761	7.22	7.205
12	8.181	8.1470	1754.77	1745.900	1.745	1.761	7.19	7.205
13	6.720	6.6395	1275.75	1267.690	0.889	0.884	7.64	7.570
14	6.559	6.6395	1259.63	1267.690	0.879	0.884	7.50	7.570
15	4.585	4.6410	782.23	766.100	1.208	1.202	8.55	8.490
16	4.697	4.6410	749.97	766.100	1.196	1.202	8.43	8.490
17	8.456	8.5785	1582.19	1590.260	2.156	2.213	7.71	7.710
18	8.701	8.5785	1598.33	1590.260	2.270	2.213	7.71	7.710
19	8.659	8.6625	66.13	64.515	0.459	0.449	4.28	4.545
20	8.666	8.6625	62.90	64.515	0.440	0.449	4.81	4.545
21	2.982	2.9435	138.70	135.475	0.811	0.799	5.43	5.665
22	2.905	2.9435	132.25	135.475	0.787	0.799	5.90	5.665
23	7.740	8.1495	624.17	629.815	0.303	0.298	6.25	6.175
24	8.559	8.1495	635.46	629.815	0.293	0.298	6.10	6.175
25	8.491	8.5925	1566.06	1566.380	0.874	0.852	7.46	7.460
26	8.694	8.5925	1566.70	1566.380	0.831	0.852	7.46	7.460
27	6.006	6.1130	2625.70	2638.605	0.765	0.769	6.76	6.780
28	6.220	6.1130	2651.51	2638.605	0.773	0.769	6.80	6.780
29	6.594	6.4540	1249.95	1234.625	0.757	0.765	7.12	7.115
30	6.314	6.4540	1219.30	1234.625	0.773	0.765	7.11	7.115
31	0.626	0.6175	0.00	0.000	0.076	0.073	5.75	5.810
32	0.609	0.6175	0.00	0.000	0.069	0.073	5.87	5.810

Table 6: Experimental design results of the biomass, proteins and glucoamylase production

		Biomass (DO 600 nm)			Glucoamylase activity (µmol/min/L)			Proteins (mg L^{-1})		
Factors		Coefficient	t. exp	Signif. (%)	Coefficient	t. exp	Signif. (%)	Coefficient	t. exp.	Signif. (%)
	b0	6.0391	195.45	<0.01***	1250.398	459.01	<0.01***	0.949	222.12	<0.01***
pH	b1	-0.3187	-10.32	<0.01***	238.492	87.55	<0.01***	0.038	8.88	<0.01***
Starch	b2	1.6914	54.74	<0.01***	372.783	136.85	<0.01***	0.124	28.99	<0.01***
Sucrose	b3	0.5289	17.12	<0.01***	-42.191	-15.49	<0.01***	-0.059	-13.72	<0.01***
NH_4Cl	b4	-0.1273	-4.12	0.0802***	128.227	47.07	<0.01***	0.003	0.81	42.9
$(NH_4)_2HPO_4$	b5	0.4686	15.16	<0.01***	114.153	41.91	<0.01***	0.116	27.18	<0.01***
NH_4NO_3	b6	0.1688	5.46	<0.01***	221.145	81.18	<0.01***	-0.030	-7.04	<0.01***
CH_4N_2O	b7	-0.1553	-5.02	0.0125***	324.900	119.27	<0.01***	0.092	21.46	<0.01***
$(NH_4)_2SO_4$	b8	-0.2911	-9.42	<0.01***	-8.018	-2.94	0.954**	-0.043	-10.00	<0.01***
Yeast extract	b9	1.2775	41.34	<0.01***	434.614	159.54	<0.01***	0.353	82.69	<0.01***
Peptone	b10	0.0517	1.67	11.4	94.660	34.75	<0.01***	0.142	33.32	<0.01***
$CaCl_2$	b11	0.1027	3.32	0.430**	-336.130	-123.39	<0.01***	0.227	53.19	<0.01***
$MnCl_2$	b12	0.1620	5.24	<0.01***	-109.215	-40.09	<0.01***	-0.059	-13.87	<0.01***
$FeCl_2$	b13	0.4253	13.76	<0.01***	-122.118	-44.83	<0.01***	-0.008	-1.79	9.2
$ZnCl_2$	b14	0.5454	17.65	<0.01***	-14.228	-5.22	<0.01***	0.128	30.03	<0.01***
$MgCl_2$	b15	0.8917	28.86	<0.01***	-46.678	-17.14	<0.01***	-0.149	-34.97	<0.01***

* Signification degree, ** High signification degree and *** Very high signification degree

source, which stimulated the growth of the cell yeasts. Earlier researchers reported similar findings wherein soluble starch was the best carbon supplement for amylase production in *Myceliophora thermophila* D14 (Sadhukhan *et al*., 1990), in *Aspergillus fumigatus* (Goto *et al*., 1998) and in *Aspergillus oryzae* (Bennamoun *et al*., 2004).

Among the nitrogen sources, yeast extract gives positive effect on the production of the biomass, followed by $(NH_4)_2HPO_4$ and NH_4NO_3. On the contrary, NH_4Cl CH_4N_2O $(NH_4)_2SO_4$ and Peptone have no effect. The effect of yeast extract on the production of biomass is very significant, because is contains amino acids and ammonium ions (NH_4), which stimulate effect on the growth (Scriban, 1993; Djekrif-Dakhmouche *et al*., 2006).

All salts tested give positive effect on the production of the biomass production. Maximum effect was given by $MgCl_2$ followed by $ZnCl_2$, $FeCl_2$, $MnCl_2$ and $CaCl_2$.

The effect of the factors on the production of Glucoamylase activity: Coefficient of determination R^2 of glucoamylase activity production is (>0.999) which strongly suggested that the fitted model could explain 99.9% of the total variation.

Variation in pH from 5 to 7 had a positive effect on glucoamylase production. These results could be explained by optimum pH for Glucoamylase activity. Mediums which record high Glucoamylase activity had their pH ranged between 6.8 and 7.8. Similar results found by Ichikawa *et al*. (2004) show that optimum pH for glucoamylase activity produced by *Thermoactinomyces vulgaris* R-47 was 6.8.

Also Starch increases the glucoamylase production, with a significant value (0.99). Therefore, the presence of starch, as enzyme substrate has inductive effect (Madihah, 2000; Murai *et al*., 1998; Hassan *et al*., 1998; Tani *et al*., 2000), its remarkable efficiency in the production of enzyme, being an inexhaustible source of carbon compared to other carbon sources (Mctigue *et al*., 1994) and because of its role in stabilizing the enzyme (Aguilar *et al*., 2000; Santamaria *et al*., 1999).

Yeast extract have highest positive effects among the nitrogen sources on the production of glucoamylase, followed by CH_4N_2O, NH_4NO_3, NH_4Cl $(NH_4)_2HPO_4$ and Peptone respectively. While $(NH_4)_2SO_4$ has a negative effect. Previous results have shown that yeast extract (Haasum *et al*., 1991; Han *et al*., 2005) ammonium nitrate (Hernández *et al*., 2006), ammonium phosphate (Jun *et al*., 2001) are good nitrogen supplements for glucoamylase production.

All tested salts give negative effect on the production of glucoamylase activity. On the basis of analyzing the results, we may suggest that microorganisms necessitate a low level of salts in order to produce enzymes because salts may be a limiting factor (Baig *et al*., 1984; Mctigue *et al*., 1994; Pedersen and Nielsen, 2000). Also, it may be explained by the fact that yeast extract provides sufficient oligoelements (Belitz and Grosch, 1987; Souci, 1994).

The effect of the factors on the production of proteins: Based on results, the coefficient of determination, R^2 was found to be 0.999, indicating that the sample variation of 99.9% can be explained by the model.

Variation of pH from 5 to 7 had a small positive effect on proteins production. These results may conclude that the optimum pH of proteins production is rather acid.

Production of proteins was stimulated by starch, with a significant value (0.99), its efficiency in the production of proteins and enzymes (Dharani Aiyer, 2004; Santos and Martins, 2003; Kiran *et al*., 2005).

Among the nitrogen sources tested, yeast extract seems to be the most influencing factor in terms of protein production; followed by Peptone $(NH_4)_2HPO_4$ and CH_4N_2O, While $(NH_4)_2SO_4$ and NH_4NO_3 had a negative effect. As reported below, yeast extract induces the production of proteins. Similar results reported that yeast extracts induce the production of proteins and enzymes (Djekrif-Dakhmouche *et al*., 2006; Teodoro and Martins, 2000).

CONCLUSION

The use of an experimental design where the main point was to reveal the influence of concentrations of macro nutrients on glucoamylase production allowed the rapid screening of large experimental domain in search of the best culture conditions for optimization of glucoamylase production. The significant achievement of the present study lies in the fact that the yeast extract, urea and starch were found to be highly significant for the enhancement of glucoamylase production. The optimization of the medium resulted in a reduced cost of medium constituent. The chosen method of optimization of medium composition was efficient, relatively simple and time and material saving.

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