International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 6, Issue 2, 2014, pp.-559-569.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

OPTIMIZATION OF THERMOPHILIC PULLULANASE AND α-AMYLASE PRODUCTION BY AMYLOLYTIC YEAST

DJEKRIF D.S.[1,2]*, GILLMANN L.[2], COCHET N.[3], BENNAMOUN L.[1], AIT-KAKI A.[1], LABBANI K.[1], NOUADRI T.[1] AND MERAIHI Z.[1]

[1]Department of Biochemistry, Faculty of Biology, Research lab in Microbiology Engineering, Biopole Chaab Erssas, Constantine University 1, 25000, Constantine, Algeria.
[2]SONAS-IUT laboratory, University of Angers, 49016, Angers, France.
[3]Research lab. Transformations in Integrated Renewable Material, University of Technology, 60205, Compiègne, Compiègne Cedex, France.
*Corresponding Author: Email- scheherazad2002@hotmail.com

Received: August 23, 2014; Accepted: September 06, 2014

Abstract- The amylolytic enzymes are the most important group of commercially produced enzymes such as α-amylase (EC 3.2.1.1) and debranching pullulanase (pullulan 6-glucanohydrolase; EC 3.2.1.41). In the present study, optimization of physical and nutritional parameters influencing pullulanase and α-amylase production attempted using the response surface methodology (RSM) from a strain of *Clavispora lusitaniae* ABS7 isolated from arid zone wheat seeds (Algerian Sahara). A Plackett-Burman design was used for screening of critical components, while the optimum selected factors are determined by the Wilson-Box design. Eight variables (agitation, pH, temperature, starch, yeast extract, $(NH_4)_2SO_4$, salts and trace-elements) were studied with the screening test. The results revealed that six factors had greater influence on the amylopullulanase production. The CCD was then used to determine the maximum amylopullulanase concentration. The optimum conditions for the highest α-amylase production (13456,36 ± 300 U) and pullulanase production (12611,6±154 were as follows: agitation 136,56 rpm, temperature 54,14°C, starch 2,66 g/l, yeast extract 0,365 g/l, salts solution 8,75 ml/l and trace-elements solution 4,3 ml/l. Also the strain grows at the optimum condition, in fermenter and produces maximally amylopullulanase on an alkaline medium.

Keywords- Thermophilic enzymes, *Clavispora lusitaniae*, Plackett-Burman design, CCD, optimization.

Citation: Djekrif D.S., et al (2014) Optimization of Thermophilic Pullulanase and α-amylase Production by Amylolytic Yeast. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 6, Issue 2, pp.-559-569.

Introduction

The global market of industrial enzymes is expected to reach 4, 4 $ billion by 2015, a compound annual growth rate of 6% over the 5-years forecast period [1]. Amylolytic enzymes constitute a class of industrial enzymes which represent approximately 30% of the world enzyme market [2].

Among these enzymes, α-amylase (endo-1, 4-α-D-glucan glucohydrolase EC 3.2.1.1) hydrolyzes the α-1, 4-linkages [3]. This enzyme is very important in biotechnology with several applications, such as clinical, medical and analytical chemistry as well as widespread application in starch saccharification and in the textile, food brewing and biorefineries [4]. Pullulanase (endo α-dextrin 6- glucanohydrolase EC 3.2.1.41), a debranching enzyme, hydrolyses the α-1, 6 linkage in pullulan and branched polysaccharides, producing maltotriose, it is an industrially important enzyme in the starch processing industry, detergent industry and other biotechnological applications [5,6]. It is possible to use pullulanase as a dental plaque control agent [7].

In the starch industry, thermostable amylases are of special interest as they could be used for saccharification processes occurring at high temperatures [8,9]. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling and increased diffusion rate [10]. It would be advantageous to have microorganisms that produce thermostable enzyme having properties of both amylase and pullulanase, because it cleaves both α-1, 4-linkages in starch and amylose and α-1,6-linkages in pullulan and branched polysaccharides, respectively [6].

Amylolytic enzymes are widely distributed in many yeast species [3]. And in recent years, research were intensified on amylolytic yeast [11,12].

Given the potential uses of α-amylases and pullulanase and the high demand for these enzymes, the need exists for the discovery of new strains of yeast that produce enzymes with novel properties and the development of low-cost, industrial-media formulation. So, we isolated amylolytic yeast from wheat seed provided from arid zone (Algerian Sahara) that was classified by molecular and biochemical criteria as a strain of *Clavispora lusitaniae*. Few studies have been done on this strain for producing enzymes.

In the present investigation, a Plackett-Burman design was used for screening, in shake flask, the important variables affecting the amylopullulanase production as well as their significance levels but

does not consider the interaction effects among the factors [13], while optimization of the selected factors for was carried out using RSM with a central composite design (CCD) of Box & Wilson [15]. The CCD and the response surface methodology (RSM) are an effective tool and have been widely used in the optimization of the fermentation process when many factors and interactions affect the desired response such as production of enzyme [14].

The aim of this work was to improve the amylopullulanase (α-amylase and pullulanase) production from *Clavispora lusitaniae* ABS7 in submerged fermentation by screening for the significant factors, and further to optimizing the levels of the screened factors. The present study is the first report that attempts to formulate a suitable medium for extracellular amylopullulanase production by *Clavispora lusitaniae* using statistical optimization methods like the Plackett-Burman design [13] and CCD using RMS [15].

Materials and Methods

Organism and Culture Conditions

Clavispora lusitaniae ABS7 was isolated from the wheat seed provided from Biskra (Algerian Sahara). This microorganism was propagated on YPSA at 40°C. For short-term storage, slants were maintained on PDA or YPGA at 4°C and stock cultures were maintained in cryo-beads at -80°C.

Medium and Cultivation

Lactoserum was provided from the ONALAIT firm, Constantine, Algeria. It was used as a base medium: its chemical composition (%) is as follows: Lactose 53, 52 ± 1, 23; crude proteins 3, 94 ± 0, 08; crude fat 0, 05 ± 0,001; and ash 1, 22 ± 0, 35. Different substances were added to the base medium for production of α-amylase, such as starch (as an enzyme substrate and inductor), yeast extract (as nitrogen and vitamins source, $(NH_4)_2SO_4$, salts solution (KH_2PO_4 850 mg/l, K_2HPO_4 150 mg/l, $MgSO_4$, $7H_2O$ 500 mg/l and $CaCl_2$ $6H_2O$ 100 mg/l) and trace-elements solution ($CuSO_4$, $5H_2O$ 40 µg/l, KI 100 µg/l, $FeCl_3$, $6H_2O$ 200 µg/l and $MnSO_4$, $4H_2O$ 400µg/l). Agitation, pH and temperature were also studied.

All the experiments were performed in 250 ml shake baffled Erlenmeyer flasks containing 50 ml of the production medium according to the design matrix (the Plackett-Burman design and the Central Composite Design). The shake flasks were sterilized at 121°C for 15 minutes. An inoculum of 2, 5 x 10^6 cellule/ml was used. The fermentation was carried out on a rotator shaker for 48 hours. At the end of the incubation period, the culture was removed and separated by a centrifugation at 8000 x g for 15 minutes. The supernatant was assayed for amylopullulanase activity.

Enzyme Assay

The extracellular α-amylase and pullulanase activities were measured by incubating 0.5 ml appropriately diluted enzyme sample with 0.5 ml of 1% (w/v) starch solution and pullulan solution in 0.1 M phosphate buffer (pH 5,5) at 40°C for 30 minutes, respectively. The reaction is stopped using 3, 5-dinitrosalicylic acid. One unit of α-amylase or pullulanase activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmoles of maltose / min [16].

Optimization Procedure

Screening of Medium Components

In order to identify the significant variables for amylopullulanase production, various components: starch, yeast extract, NH_4SO_2, salts, trace-elements and cultivation parameters (agitation, pH and temperature) were tested and identified via the Plackett-Burman design experiment. A total of eight variables and three dummy variables were screened in 12 trials [Table-1].

Table 1- Plackett-Burman experimental design matrix with amylopullulanase production.

Experiments	Variables											a-amylase Activity (IU)	Pullulanase Activity (IU)
	A	B	C	(D)	E	F	G	(H)	I	J	(K)		
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	1	-1	6693,04	6490,16
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	8591,02	8380,00
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	8227,60	8022,60
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	9044,67	8976,60
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	8770,76	8530,12
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	8423,00	8212,00
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	6434,69	6201,00
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	7335,81	7098,00
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	8181,70	7959,16
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	9222,20	9000,50
11	+1	-1	+1	+1	1	-1	-1	-1	+1	-1	+1	8598,78	8389,04
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8809,00	8616,00

Notes: A, B, C, E, F, G, I and J are assigned variables; D, H and K are dummy (unassigned variables); IU: µMoles of maltose per min.

Each variable was represented at two levels: high (+1) and low (-1). The low and high levels of these variables were taken as agitation (50 and 200 rpm), pH (3 and 6), temperature (30 and 50°C), starch (0 and 10 g/l), yeast extract (0 and1 g/l), $(NH_4)_2SO_4$ (0 and 1 g/l), salts (0 and 10 ml/l), and trace elements (0 and 10 ml/l).

The effect of each variable was determined by the [Eq-1]:

$$E(Xi) = 2(\sum Mi + - \sum Mi - / N) \quad (1)$$

where E (xi) is the effect of parameter under study, Mi+ and Mi- are the amylopullulanase activities (responses) of trials at which the variable was at its higher and lower levels respectively and N is the number of trials (12).

The reaction of the explicated variables, after experimentation, is adjusted to the following first degree model [Eq-2]:

$$Y = Const\tan t + \sum \beta iXi + error) \quad (2)$$

where βi represents the regression coefficients and Xi represents the explicative variables.

The estimations of the least squares of these coefficients are determinate from the student t-test and P signification probability. Then, all coefficients having a signification probability lower than 70% level are rejected, and the corresponding variables are considered without effect on the reaction. The statistical software package Minitab ver. 17 (Minitab Inc. USA) was used to analyze the experimental design.

Optimization of Screened Components

The levels of six independent variables - agitation (A), temperature (C), starch (E), yeast extract (F), salts solution (I) and trace-elements solution (J) - selected by Plackett and Burman's design were optimized by the central composite design (CCD) under the response surface methodology (RSM). Each factor in the design was studied at five levels [Table-2].

A set of 82 experiments was used [Table-3]. All variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used, and the full experimental design with respect to their values in coded form is listed in [Table-3]. Upon completion of the experiments, the average of the α-amylase production was taken as the dependent variable or response (Y).

Table 2- Coded and uncoded values of experimental variables used in the central composite design

Variables	Symbol code	Levels				
		-α (-2,828)	-1	0	1	+α (+2,828)
Speed (rpm)	A	23,44	60	80	100	136,56
Temperature (°C)	C	25,86	35	40	45	54,14
Starch (g/l)	E	1,172	3	4	5	6,828
Yeast extract (g/l)	F	0,117	0,3	0,4	0,5	0,682
Salts solution (ml/l)	I	4,344	8	10	12	15,65
Trace-elements (ml/l)	J	1,172	3	4	5	6,828

Table 3- The experimental design and results of CCD for the study of six variables

Exp. no.	Variables						Activity (IU)	
	A	C	E	F	I	J	α-amylasic	pullulytic
Fractional 2^6 factorial design								
1	-1	-1	-1	-1	-1	-1	10589,5	10213,5
2	+1	-1	-1	-1	-1	-1	11071,7	10695,7
3	-1	+1	-1	-1	-1	-1	10816,7	10440,7
4	+1	+1	-1	-1	-1	-1	11664,7	11288,7
5	-1	-1	+1	-1	-1	-1	11003,3	10627,3
6	+1	-1	+1	-1	-1	-1	10836,8	10460,8
7	-1	+1	+1	-1	-1	-1	10822,8	10446,8
8	+1	+1	+1	-1	-1	-1	11566,0	11190,0
9	-1	-1	-1	+1	-1	-1	10664,0	10288,0
10	+1	-1	-1	+1	-1	-1	10630,0	10254,0
11	-1	+1	-1	+1	-1	-1	11226,8	10850,8
12	+1	+1	-1	+1	-1	-1	11648,5	11272,5
13	-1	-1	+1	+1	-1	-1	10869,5	10493,5
14	+1	-1	+1	+1	-1	-1	10854,0	10478,0
15	-1	+1	+1	+1	-1	-1	11407,7	11031,7
16	+1	+1	+1	+1	-1	-1	11540,0	11164,0
17	-1	-1	-1	-1	+1	-1	10625,1	10249,1
18	+1	-1	-1	-1	+1	-1	10654,7	10278,7
19	-1	+1	-1	-1	+1	-1	10908,4	10532,4
20	+1	+1	-1	-1	+1	-1	11405,0	11029,0
21	-1	-1	+1	-1	+1	-1	11163,6	10787,6
22	+1	-1	+1	-1	+1	-1	10844,6	10468,6
23	-1	+1	+1	-1	+1	-1	10874,2	10498,2
24	+1	+1	+1	-1	+1	-1	11661,0	11285,0
25	-1	-1	-1	+1	+1	-1	10127,1	9751,1
26	+1	-1	-1	+1	+1	-1	10413,5	10037,5
27	-1	+1	-1	+1	+1	-1	10885,2	10509,2
28	+1	+1	-1	+1	+1	-1	11277,0	10901,0
29	-1	-1	+1	+1	+1	-1	10913,1	10537,1
30	+1	-1	+1	+1	+1	-1	10669,4	10293,4
31	-1	+1	+1	+1	+1	-1	10934,9	10558,9
32	+1	+1	+1	+1	+1	-1	11197,6	10821,6
33	-1	-1	-1	-1	-1	+1	10234,5	9858,5
34	+1	-1	-1	-1	-1	+1	10687,4	10311,4
35	-1	+1	-1	-1	-1	+1	11451,6	11075,6
36	+1	+1	-1	-1	-1	+1	11627,9	11251,9
37	-1	-1	+1	-1	-1	+1	10657,7	10281,7
38	+1	-1	+1	-1	-1	+1	10460,2	10084,2
39	-1	+1	+1	-1	-1	+1	11675,7	11299,7
40	+1	+1	+1	-1	-1	+1	11491,9	11115,9

Table 3- Continue..

Exp. no.	Variables						Activity (IU)	
	A	C	E	F	I	J	α-amylasic	pullulytic
Fractional 2^6 factorial design								
41	-1	-1	-1	+1	-1	+1	10806,0	10430,0
42	+1	-1	-1	+1	-1	+1	10788,3	10412,3
43	-1	+1	-1	+1	-1	+1	11520,0	11144,0
44	+1	+1	-1	+1	-1	+1	11959,0	11583,0
45	-1	-1	+1	+1	-1	+1	10911,5	10535,5
46	+1	-1	+1	+1	-1	+1	10703,0	10327,0
47	-1	+1	+1	+1	-1	+1	11859,5	11483,5
48	+1	+1	+1	+1	-1	+1	11265,5	10889,5
49	-1	-1	-1	-1	+1	+1	10541,8	10261,2
50	+1	-1	-1	-1	+1	+1	10516,2	10286,8
51	-1	+1	-1	-1	+1	+1	11285,1	10909,1
52	+1	+1	-1	-1	+1	+1	11989,0	11613,0
53	-1	-1	+1	-1	+1	+1	11022,0	10646,0
54	+1	-1	+1	-1	+1	+1	10530,2	10154,2
55	-1	+1	+1	-1	+1	+1	11821,2	11445,2
56	+1	+1	+1	-1	+1	+1	11687,4	11311,4
57	-1	-1	-1	+1	+1	+1	10763,7	10387,7
58	+1	-1	-1	+1	+1	+1	10150,5	10652,5
59	-1	+1	-1	+1	+1	+1	11456,3	11080,3
60	+1	+1	-1	+1	+1	+1	11668,7	11292,7
61	-1	-1	+1	+1	+1	+1	11460,9	11084,9
62	+1	-1	+1	+1	+1	+1	10674,9	10298,9
63	-1	+1	+1	+1	+1	+1	12013,2	11637,2
64	+1	+1	+1	+1	+1	+1	11191,7	10815,7
Star points								
65	-α	0	0	0	0	0	12049,8	11673,8
66	+α	0	0	0	0	0	11839,4	11463,4
67	0	-α	0	0	0	0	10056,0	9680,0
68	0	+α	0	0	0	0	11759,4	11483,4
69	0	0	-α	0	0	0	10248,5	9872,5
70	0	0	+α	0	0	0	10634,5	10258,5
71	0	0	0	-α	0	0	10332,5	9956,5
72	0	0	0	+α	0	0	10561,3	10185,3
73	0	0	0	0	-α	0	10653,2	10277,2
74	0	0	0	0	+α	0	10362,1	9986,1
75	0	0	0	0	0	-α	10186,0	9810,0
76	0	0	0	0	0	+α	10676,5	10300,5
Central points								
77	0	0	0	0	0	0	11821,9	11445,9
78	0	0	0	0	0	0	11984,0	11608,0
79	0	0	0	0	0	0	11607,2	11231,2
80	0	0	0	0	0	0	11807,9	11431,9
81	0	0	0	0	0	0	11572,3	11196,3
82	0	0	0	0	0	0	11789,6	11413,6

Note; α= 2,828.

Statistical Analysis and Modeling

The data obtained from CCD on the α-amylase and pullulanase production were subjected to the analysis of variance (ANOVA). The results of CCD were used to fit a second order polynomial equation [Eq-1], as it represents the behaviour of such a system more appropriately:

$$Y = \beta 0 + \sum \beta iXi + \sum \beta iiXii + \sum \beta ijXiXj \qquad (3)$$

where Y represents response variable, β0 is the regression coefficient at the center point (offset term); βi is the linear coefficients, βii, the squared coefficient, βij is the interaction coefficient, and XiXj are the level of the independent variables which influence the response.

The developed regression model was evaluated by analyzing the values of the regression coefficients, analysis of variance (ANOVA), p- and F-values. The statistical significance of the model equation was determined by Fisher's test value, and the coefficient of determination R^2. The data analysis, the optima determination and the generation of the response surface graphics were done by Minitab 17 software.

Results and Discussion

Screening of the Important Medium Components

To enhance the production of the amylopullulanase, statistical method of medium optimization was conducted. Statistical analysis

using a Plackett-Burman design indicated that agitation (A), temperature (C), starch (E), yeast extract (F), salts (I) and trace elements (J) were significantly affected the amylopullulanase production, with P-values less than 0,05 and confidence level above 70%. For both enzymes production, the confidence level of variables pH (B) and NH_4Cl (G) were below 70% with P-values above 0, 05 and hence considered insignificant. Statistical analysis of the responses was performed, as shown in [Table-4].

The reduced polynomial equations for α-amylase production (Y1) and pullulanase (Y2) may be written as follows:

$$Y1 = 8194,4 - 615,8\ A + 404,3\ C - 172,2\ E - 187,5\ F + 198,1\ I - 240,1\ J \quad (4)$$

$$Y2 = 7989,6 - 629,6\ A + 390,6\ C - 161,8\ E - 200,8\ F + 204,6\ I - 257,4\ J \quad (5)$$

The production of these enzymes is better explained using the explicative factors (variables) A, C, E, F, I and J since their coefficients are the most important ones and because of the high signification level [Table-4]. The correlation between A, E, F and J and the production of enzyme is negative. On the other hand, it is positive between C, I and the production of α-amylase.

Table 4- Statistical analysis from the results of Plackett-Burman design for amylopullulanase.

Code	Factors	Effect	Coefficient	T (xi)	p-value	Confidence level (%)
			α-amylase			
	Constant		8194,4	71,48	0,000	
A	Agitation	-1231,5	-615,8	-5,37	0,013	98,7
B	pH	-32,6	-16,3	-0,14	0,893	10,7
C	Temperature	808,6	404,3	3,53	0,039	96,1
D	Error	-	-	-	-	
E	Starch	-344,4	-172,2	-1,50	0,230	77
F	Yeast extract	-375,0	-187,5	-1,64	0,200	80
G	$(NH_4)_2SO_4$	-87,8	-43,9	-0,38	0,727	27,3
H	Error	-	-	-	-	
I	Salts	396,2	198,1	1,73	0,182	81,8
J	Trace-elements	-480,2	-240,1	-2,09	0,127	87,3
K	Error	-	-	-	-	
			Pullulanase			
	Constant		7989,6	62,48	0,000	
A	Agitation	-1259,2	-629,6	-4,91	0,016	98,4
B	pH	-11,1	-5,5	-0,04	0,968	03,2
C	Temperature	7818,3	390,6	3,05	0,056	94,4
D	Error	-	-	-	-	
E	Starch	-323,6	-161,8	-1,26	0,296	70,46
F	Yeast extract	-401,6	-200,8	-1,57	0,215	78,5
G	$(NH_4)_2SO_4$	-62,1	-31,0	-0,24	0,827	17,3
H	Error	-	-	-	-	
I	Salts	409,1	204,6	1,60	0,209	79,1
J	Trace-elements	-514,9	-257,4	-2,01	0,138	86,2

Effect of Carbon Source

The carbon source is an important factor affecting enzyme production, especially when the carbon source also plays an important role in the enzyme induction. The addition of starch shows a significantly negative effect on α-amylase (P≥77%) and pullulanase production (P≥70, 4%), which leads us to conclude that above a certain concentration of carbon substrate; catabolic repression may occur [17]. This repression may occur due to limitation of other media components in the culture medium. Also, the lactoserum provides lactose as a carbon source. It was found that beyond 5g/L, starch inhibits yeast growth and amylolytic activity [17]. Many other works, reported starch as the best carbon source for the production of α-amylase by bacterial or fungal strains [18, 19]. It is because α-amylase is an extracellular enzyme and its production is increased by its substrate. Also for, its inductive effect [20] and, its role in stabilizing the enzyme [21].

For pullulanase, the presence of α-(1, 6)-linkages in polysaccharides complex can induce its production. Maximum production of pullulanase was observed with soluble starch, it is therefore a good source of carbon for production of pullulanase and it can induce its release. It was found that, at concentration above 1% starch, the productivity for the extracellular enzymes (α-amylase and pullulanase) was reduced [22].

Effect of Nitrogen Source

The results reveal that NH_2SO_4 did not affect α-amylase and pullulanase production in the medium. However, α-amylase and pullulanase activity seemed to be induced by yeast extract. The yeast extract has a significantly negative effect on the production of α-amylase (P≥80%) and on the production of pullulanase (P≥78,5%) and, in this way, causes a decrease of 375 U and 401,64 U for α-amylase and pullulanase respectively. This result is probably due to the excessive amount of the yeast extract, which may inhibit the production of enzyme when concentration exceeds a critical value [23]. The decrease in amylase production at higher concentrations of beef extract could be due to the induction of proteases resulting in destruction of amylolytic enzymes [18]. On the other hand, it was

reported that yeast extract was the more efficient source of nitrogen and an inducer for pullulanase and α-amylase production [19, 24].

Effect of Salts and Oligo-traces

Salts (KH_2PO_4, K_2HPO_4, $MgSO_4$, $7H_20$ and $CaCl_2$, 6 H_2O) have a significantly positive effect on the production of α-amylase and pullulanase (P≥81, 81% and 79, 1% respectively) causing an increased activity of 396, 2 U for α-amylase and 409, 1 U for pullulanase. The effect of trace- elements on the production of α-amylase and pullulanase is significantly negative (P≥87,3% and 86,2% respectively) and causes a decrease of α-amylase activity of 480, 2 U and of pullulanase of 514,9 U. These results are probably due to the excessive amount of trace-elements because the lactoserum, also, provides trace-elements that are necessary for the yeast's growth and, consequently, for the enzymes synthesis. Alpha amylase is known to be a calcium metalloenzyme and the production of α-amylase is Ca^{2+} dependent [25]. The Ca^{2+} ions are equally important for the α-amylase and pullulanase activities, for their thermal stability and for maintaining space conformation of the enzymes [26,27]. Mrudula (2010) revealed the highly significant effect of Mg^{2+} on the production of α-amylase and pullulanase [19,26]. It played an important role on production of α-amylase and pullulanase which has been reduced to 50% when Mg^{2+} was omitted from the medium [3].

Phosphate plays an important regulatory role in the synthesis of primary and secondary metabolites in microorganisms [28]. It was found that phosphate ions may not only support the growth of microorganism but also act as stimulators of alpha amylase and pullulanase [6,9,26]. It was reported that Mn^{2+}, Zn^{2+} and Cu^{2+} increased enzyme production [8,19,26].

Effect of Temperature

The variation of temperature from 30°C to 50°C leads to a positive effect (P≥96,1% and P≥94,4%) on the production of the α-amylase and pullulanase respectively, which causes an increased activity of 808,6 U for α-amylase and 781,3 U for pullulanase. Generally, the optimal temperature of the production of enzymes from yeast is between 25°C and 30°C. The dissimilarity between our enzyme and this one reported in the literature may be attributed to strain variation, the structure of the enzyme, the conditions of the environment and the strain source because *Clavispora lusitaniae* ABS7 was isolated from the wheat seed provided from the Algerian Sahara.

Microorganisms capable of growing optimally at temperatures between 50°C and 60°C are designated as moderate thermophiles. It can be assumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, may be secondarily adapted to life in hot environments [29].

Effect of Agitation

The speed of agitation is a significantly negative effect (P≥98, 7% for α-amylase and 98, 4% for pullulanase) and decreases the α-amylase activity of 1231, 5 U and the pullulanase activity of 1259, 2 U. Then a decrease of the speed of agitation from 200 rpm to 50 rpm appears necessary for increasing the production of α-amylase and pullulanase. Agitation speed is beneficial for good mixing throughout the fermentation, which ensures sufficient oxygen transfer in aerobic culture, and consequently improves the cell growth and metabolite synthesis. But too high agitation speed results in intensive shear forces, and in turn causes damage to cell structure and decrease in the yield of secondary metabolite [30].

Optimization of Screened Medium Components

Statistical Analysis

The coefficients of regression equations were calculated using the Minitab17 software [Table-5],[Table-6]. The significance of each coefficient was determined by t-values and the corresponding p values that are listed in [Table-6]. The values of p that are less than 0, 05 indicate that the model terms are significant. The larger value of t-test and the smaller value of p-value indicate the high signification of the corresponding coefficient and the insignificant terms were omitted.

Table 5- Coefficient of regression of the equations representing the production of α- amylase.

Variable	Effect	Coefficient of regression	Standard error	t-value	p-value
Constant		11790,5	± 66,8	1,76,472	0
β_1	100,3	50,2	± 55,9	0,9	0,373
β_2	1964,1	982	± 55,9	17,57	**0,000**[a]
β_3	397,5	198,8	± 55,9	3,56	**0,001**[a]
β_4	64,4	32,2	± 55,9	0,58	0,567
β_5	-198,5	-99,3	± 55,9	-1,78	0,081
β_6	459,1	229,5	± 55,9	4,11	**0,000**[a]
β_{11}	388	194	± 144	1,35	**0,182**
β_{22}	-1685	-843	± 144	-5,87	**0,000**[a]
β_{33}	-2618	-1309	± 144	-9,11	**0,000**[a]
β_{44}	-2607	-1304	± 144	-9,07	**0,000**[a]
β_{55}	-2486	-1243	± 144	-8,65	**0,000**[a]
β_{66}	-2638	-1319	± 144	-9,18	**0,000**[a]
β_{12}	1437	718	± 177	4,07	**0,000**[a]
β_{13}	-1621	-811	± 177	-4,59	**0,000**[a]
β_{14}	-1097	-548	± 177	-3,10	**0,003**[a]
β_{15}	-635	-318	± 177	-1,80	0,078
β_{16}	-1547	-774	± 177	-4,38	**0,000**[a]
β_{23}	-1022	-511	± 177	-2,89	**0,005**[a]
β_{24}	86	43	± 177	0,24	0,809
β_{25}	-148	-74	± 177	-0,42	0,677
β_{26}	1787	893	± 177	5,06	**0,000**[a]
β_{34}	108	54	± 177	0,31	0,761
β_{35}	863	432	± 177	2,44	**0,018**[b]
β_{36}	-142	-71	± 177	-0,40	0,688
β_{45}	-931	-466	± 177	-2,64	**0,011**[b]
β_{46}	690	345	± 177	1,95	0,056
β_{56}	832	416	± 177	2,36	**0,022**[b]

Notes: [a]P<0, 01 indicate the model terms are highly significant.
[b]P<0, 05 indicate the model terms are significant.
Determination Coefficient R^2= 0.9291, Predicted R^2= 0, 8364 and Adjusted R2= 0.8936.

The following regression equations were obtained:

$$Y1 = 11790,5 + 982C + 198,8E + 229,5J - 843C^2 - 1309E^2 - 1304F^2 - 1243I^2 - 1319J^2 + 718AC - 811AE - 548AF - 774AJ - 511CE + 893CJ + 466FI + 416IJ \quad (6)$$

$$Y2 = 11421 + 952,4C + 159,2E + 269,1J - 789C^2 - 1306E^2 - 1300F^2 - 1240I^2 - 1316J^2 + 602AC - 927AE - 445AF - 658AJ - 371CE + 753CJ - 386FI + 425FJ + 556 IJ \quad (7)$$

where Y1 is the α- amylase activity, Y2 is the pullulanase activity and A, C, E, F, I and J are coded values of the test variables, agita-

tion (rpm), temperature (°C), starch (g/l), yeast extract (g/l), salts solution (ml/l) and trace-elements solution (ml/l) respectively.

Table 6- Coefficient of regression of the equations representing the production of pullulanase.

Variable	Effect	Coefficient of regression	Standard error	t-value	p-value
Constant		11421	± 69,5	164,43	0.000
β_1	166	83	± 58,1	1,43	0,159
β_2	1904,9	952,4	± 58,1	16,39	**0,000**[a]
β_3	318,4	159,2	± 58,1	2,74	**0,008**[a]
β_4	109,3	54,7	± 58,1	0,94	0,351
β_5	-119,3	-59,7	± 58,1	-1,03	0,309
β_6	538,3	269,1	± 58,1	4,63	**0,000**[a]
β_{11}	395	197	± 149	1,32	0,192
β_{22}	-1579	-789	± 149	-5,29	**0,000**[a]
β_{33}	-2611	-1306	± 149	-8,74	**0,000**[a]
β_{44}	-2601	-1300	± 149	-8,71	**0,000**[a]
β_{55}	-2479	-1240	± 149	-8,30	**0,000**[a]
β_{66}	-2632	-1316	± 149	-8,81	**0,000**[a]
β_{12}	1205	602	± 184	3,28	**0,002**[a]
β_{13}	-1853	-927	± 184	-5,05	**0,000**[a]
β_{14}	-890	-445	± 184	-2,42	**0,019**[b]
β_{15}	-403	-202	± 184	-1,10	0,277
β_{16}	-1315	-658	± 184	-3,58	**0,001**[a]
β_{23}	-742	-371	± 184	-2,02	**0,048**[b]
β_{24}	-73	-37	± 184	-0,20	0,843
β_{25}	-428	-214	± 184	-1,16	0,249
β_{26}	1507	753	± 184	4,10	**0,000**[a]
β_{34}	-51	-25	± 184	-0,14	0,890
β_{35}	583	292	± 184	1,59	0,118
β_{36}	-422	-211	± 184	-1,15	0,255
β_{45}	-772	-386	± 184	-2,10	**0,040**[b]
β_{46}	849	425	± 184	2,31	**0,025**[b]
β_{56}	1112	556	± 184	3,03	**0,004**[a]

Notes: [a] P<0, 01 indicate the model terms are highly significant.
[b] P<0, 05 indicate the model terms are significant.
Determination Coefficient R^2= 0.9201, Predicted R^2= 0, 8152 and Adjusted R^2= 0.8801.

To test the goodness of fit of the model, the determination coefficient R^2 was evaluated. The models presented a high determination coefficient R^2 = 0, 9291 (for α-amylase production) and R^2 = 0, 9201 (for pullulanase production), which indicates that 92, 9% and 92% of the total variations are explained by the two models, respectively [Table-5],[Table-6]. The predicted R^2 of 0, 8364 for α-amylase and 0, 8152 for pullulanase were in reasonable agreement with the adjusted R^2 of 0, 8936 and 0, 8801 (for α-amylase and pullulanase respectively). This indicated a good agreement between the experimental and predicted values for the α-amylase and pullulanase production [31].

The results were analyzed using the analysis of variance (ANOVA) as being appropriate to the experimental design used [Table-7]. The ANOVA of the quadratic regression model demonstrates that the model for α-amylase and pullulanase activities are highly significant, as is evident from the model F-value corresponding to α-amylase and pullulanase was 26, 20 and 23, 03 respectively and a very low probability value p=0,000 for both enzymes.

This indicates that the combined effects of all the independent variables significantly contributed to the enhancement of the α-amylase and pullulanase production. Also, the F-value for inadequate adjustment was 1, 39 for α-amylase and 1, 51 for pullulanase [Table-7]. The high F-value and non significant inadequate adjustment indicate that the model is a good fit. The P-value for the model (0,000) (for both enzyme) and inadequate adjustment (0, 390 and 0,347 for α- amylase and pullulanase respectively) also suggested that the obtained experimental data was a good fit with the model.

Table 7- ANOVA for quadratic model of α-amylase and pullulanase.

Source	dl	SS	MS	F	p-value
α-amylase					
Model	27	22096378	818384	26,20	0,000
Linear	6	10702626	1783771	57,10	0,000
Square	6	7447566	1241261	39,74	0
Interaction	15	3946187	263079	8,42	0
Error residual	54	1686775	31237		
Inadequate adjustment	49	1571105	32063	1,39	0,390
Total	81	23783153			
Pullulanase					
Model	27	20988487	777351	23,03	0,000
Linear	6	10186022	1697670	50,29	0,000
Square	6	7385919	1230986	36,46	0
Interaction	15	3416546	227770	6,75	0
Error residual	54	1823051	33760		
Inadequate adjustment	49	1707381	34845	1,51	0,347
Total	81	22811537			

Table 8- Optimal solutions of the coded coordinates and the corresponding actual values.

Variables	Level before optimization	Levels after optimization	Before optimization	After optimization	
				Predicted	Experimental
A : Agitation (rpm)	200	136,56		α-amylase production (IU)	
C : Temperature (°C)	30	54,14	6639,16	13128	13456,36 ±300
E : Starch (g/l)	10	2,66			
F: Yeast extract (g/l)	1	0,365		Pullulanase production (IU)	
I: Salts (ml/l)	0	8,75	6308,5	12825,1	12611,6±154
J: Trace-elements (ml/l)	0	4,3			

Response Surface Plotting

A fitted response surface on the α-amylase and pullulanase production was generated from [Eq-6] and [Eq-7] using the Minitab version as shown in [Fig-1],[Fig-2]. The 2D contour plots are the graphical representations of the regression equation. Each contour curve represents an infinitive number of combinations of two test variables with the other four maintained at their respective zero level. Elliptical contours are obtained when there is a perfect interaction between the independence variable [14].

The contour plots visually interpret the interaction between the two

variables and facilitate the location of optimum experimental conditions. The shadowed zones dark determine the conditions that maximize the production of enzyme.

[Fig-1](A) and [Fig-2](A) show the effect of agitation and temperature on α-amylase and pullulanase production, there is a strong interaction between agitation and temperature (p=0,000 for α- amylase and 0,002 for pullulanase) [Table-1],[Table-2], where the α-amylase and pullulanase production increased with increasing both temperature and agitation. Therefore, a temperature of 54, 14°C and agitation of 135, 56 rpm was considered favourable for maximum yield of α- amylase and pullulanase.

The results showed the α-amylase and pullulanase production which was considerably affected by varying the agitation and the concentration of starch [Fig-1](B) and [Fig-2](B). The 2D plot and also a strong P-value (0,000) show that there is a significant interaction between the two variables. The coefficient estimated for these interaction terms has a negative sign (β13= -811 and -927 for α-amylase and pullulanase respectively) and may include that for an increase of the response, the coded levels of agitation and starch must not have the same sign; which means that when there is increase in agitation, there must be decrease in starch; and at a higher starch concentration, the maximum agitation shifts towards a lower value.

[Fig-1](C) and [Fig-2](C) show the negative effect of the interaction between agitation and yeast extract on the α-amylase and pullulanase production, which increased by increasing agitation and decreasing yeast extract. So the maximum of enzyme production was obtained only at high levels of agitation (136, 56 rpm) and low levels of trace elements (approximately 4 ml/l).

Similarly, [Fig-1](D) and [Fig-2](D) show the negative effect of the interaction of agitation and trace-elements, on the production of enzymes that increased by increasing agitation and decreasing the trace-elements concentration, or vice versa.

The elliptical contours obtained [Fig-1](E) and [Fig-2](E) indicate a perfect interaction between the temperature and the starch p=0,005 [Table-5],[Table-6]. The predicted α-amylase production increased at the higher values of temperature and lower values of starch concentration. The maximum production of α-amylase (> 12000 UI) was predicted at the temperature approximately of 54°C and the starch concentration approximately 3 g/l.

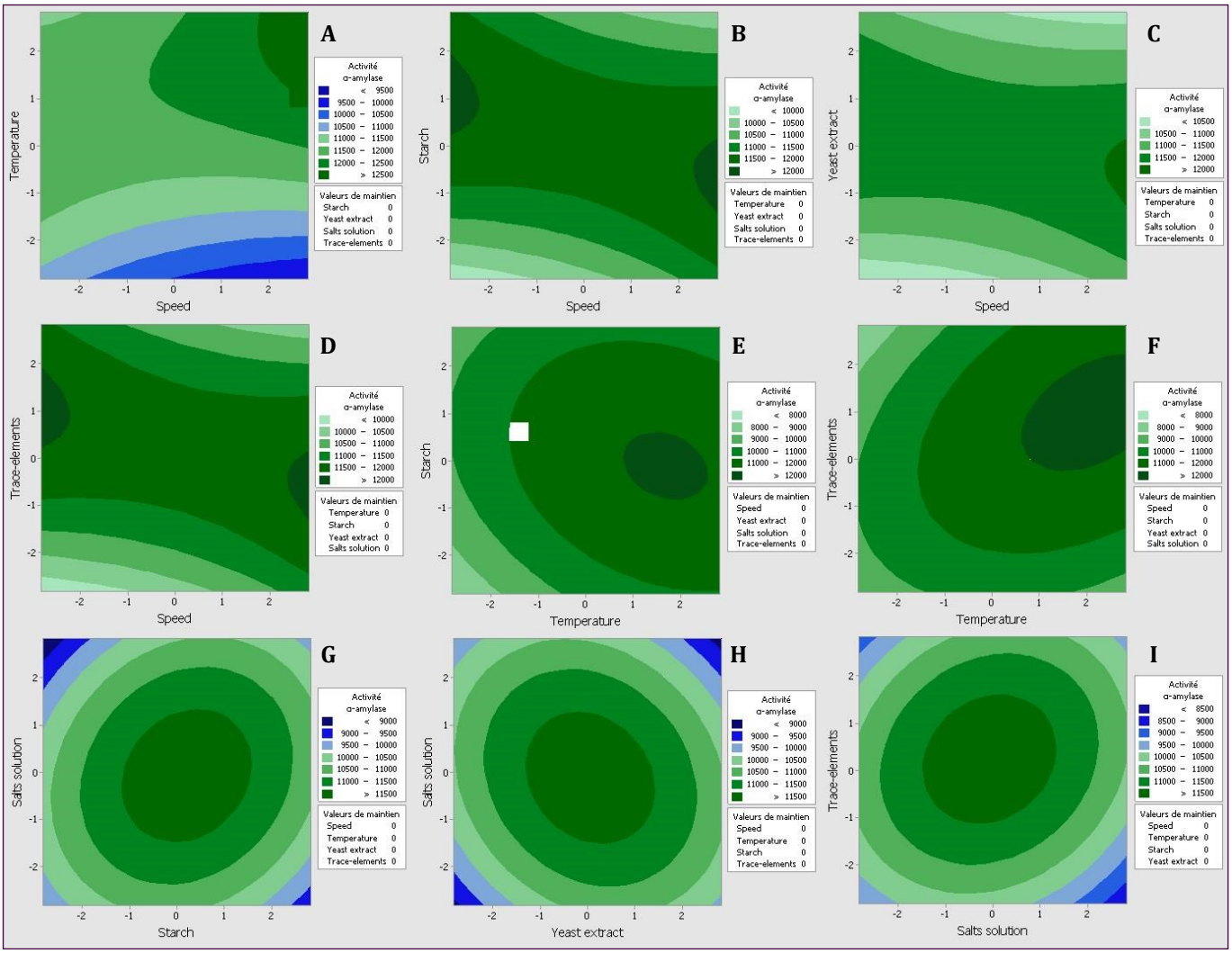

Fig. 1- Response surface plot for the production of thermostable alpha amylase by *Clavispora lusitaniae* ABS7: **(A)** Effect of agitation and temperature; **(B)** Effect of agitation and starch; **(C)** Effect of agitation and yeast extract; **(D)** Effect of agitation and trace elements; **(E)** Effect of temperature and starch; **(F)** Effect of temperature and trace elements; **(G)** Effect of starch and salts; **(H)** Effect of yeast extract and salts; **(I)** Effect of Salts- trace elements.

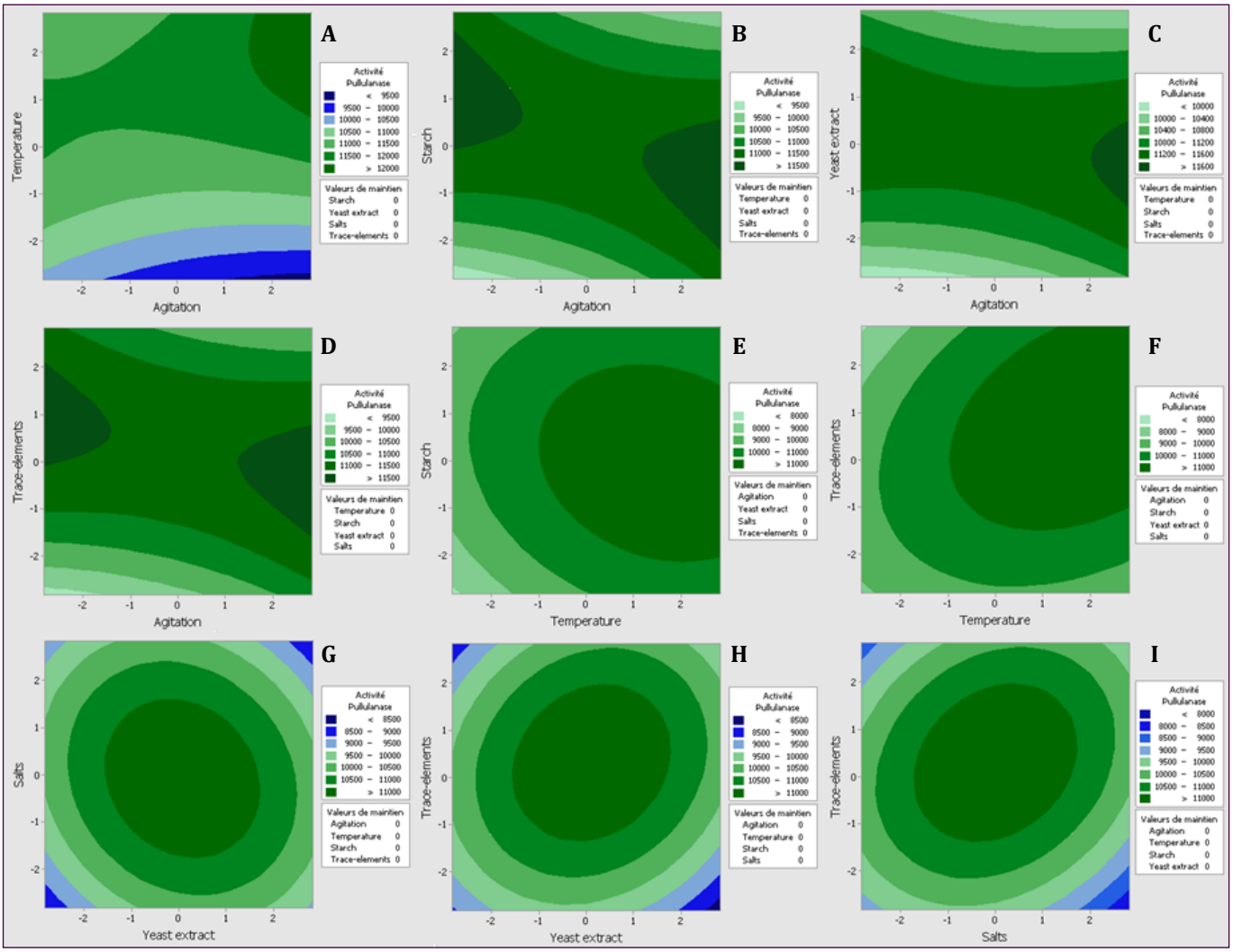

Fig. 2- Response surface plot for the production of thermostable pullulanase by *Clavispora lusitaniae* ABS7: **(A)** Effect of agitation and temperature; **(B)** Effect of agitation and starch; **(C)** Effect of agitation and yeast extract; **(D)** Effect of agitation and trace elements; **(E)** Effect of temperature and starch; **(F)** Effect of temperature and trace elements; **(G)** Effect of yeast extract and salts; **(H)** Effect of yeast extract and trace elements; **(I)** Effect of Salts- trace elements.

Temperature and trace-elements are the important factors for the α-amylase and the pullulanase production, p= 0,000 [Table-6]. Hence, a strong interaction between them for the α-amylase and pullulanase production is inevitable. The culture temperature (approximately 54°C) and trace-elements (approximately 4, 5 ml/l) appear to the most favourable for maximum α-amylase and pullulanase production [Fig-1](F) and [Fig-2](F).

[Fig-1](G) depicts a contour plot of a calculated response surface from the interaction between the starch and salts concentration. It is indicate that α-amylase production was drastically affected by a slight change in the level of these two factors. The higher and lower concentration of both variables resulted in lesser enzyme production but mid-levels provide a maximum yield.

[Fig-1](H) and [Fig-2](G) show the elliptical response surface plot of α-amylase and pullulanase production as a function of yeast extract and salts concentrations. The predicted enzyme production decreased at the higher, and the lower value of ranges for both yeast extract and salts concentrations.

The elliptical nature of the contour plots indicates that the mutual interaction between the independent variables yeast extract and trace-elements were significant [Fig-2](H). Maximum pullulanase activity, in these conditions, was predicted > 11000 UI.

The 2D plot in [Fig-1](I) and [Fig-2](I) and also p values (0,022 for α -amylase and 0,004 for pullulanase) [Table-5],[Table-6] show that there is a significant interaction between salts and trace-elements. The results show that the α-amylase and pullulanase production were considerably affected by varying the concentration of salts and trace-elements. The maximum production was obtained at the intersection point of the ellipse's major and minor axes. Production decreases at the maximum and minimum values of ranges for both parameters.

Minitab was used to solve the regression equations [Eq-6] and [Eq-7]. The optimal values of the test variables in uncoded (actual value) units were: agitation (136, 56 rpm), temperature (54, 14 °C), starch (2, 66 g/l), yeast extract (0,365 g/l), salts (8, 75 ml/l) and trace-elements (4, 3 ml/l)

Model Validation

The study of the amylopullulanase production was performed on the optimized medium in shake baffled Erlenmeyer flasks and a 2.5 l

fermenter. The maximum production of amylopullulanase using a statistical model is 13 456.36 ± 300 IU for α-amylase and 12611, 6 ±154 IU for pullulanase respectively (in batch (Erlenmeyer flasks 250 ml). This is obviously in close agreement with the model prediction which is 13231 IU for α-amylase and 12825, 5 IU for pullulanase. After optimization, the amylopullulanase production has doubled [Table-8].

A comparative study of the production of amylopullulanase was performed on the optimized medium in Erlenmeyer flasks and a 2.5 l fermenter. The results are shown in [Fig-3].

Testing the *Clavispora lusitaniae* ABS7 yeast, in optimized conditions in a base medium of lactoserum, gives a maximum alpha amylase production of 22038 IU, and pullulanase production of 20716 IU in a 2,5 L fermenter after 28h of fermentation at 54°C and pH8 [Fig-3]. Maximum production in shake flask was obtained after 40 h of incubation and in the fermenter was reached after 28H. This time -saving of 70% is justified by good control of the fermentation conditions. This high production of amylopullulanase by *Clavispora lusitaniae* ABS7 yeast in an appreciable time also proves the validity of the model.

The enzyme production increased in a laboratory fermenter. The reduction in the enzyme production time and enhancement in enzyme secretion in the bioreactor has been attributed to uniform distribution of nutrients, improved aeration [32]. Improvements in product yields are expected in the fermenter due to better control of process parameters [33]. Usually the pH of the alpha-amylase production in most yeast is between and 7: It is 5 in *Candida utilis* NOY1 [11], 5.5 for *Saccharomycopsis fibuligera* DSM-70554 yeast [34], between 5.5 and 7 in *Schwanniomyces castellii* [35] and 6.5 in *Candida antractica* CBS 6678 [21]. That of the pullulanase of the *Aureobasidium pullulans* is 5, 5 [12]. The pH of maximum production of the enzymes in the yeast of our study is 7, 82 [Fig-3]. This singularity will certainly influence the physico-chemical properties of the enzyme. However, a strain of *Aspergillus niger* SK01 isolated from sea water has a strongly alkaline pH of production (pH 10) and an optimum production of much higher temperature (70°C) [36], while it is recognized that *Aspergillus niger* is mesophilic and grows in an acidic medium (pH between 5 and 6). Then, we conclude that the ecological niche of microorganisms has an influence on enzyme production (pH, temperature, amount of enzyme).

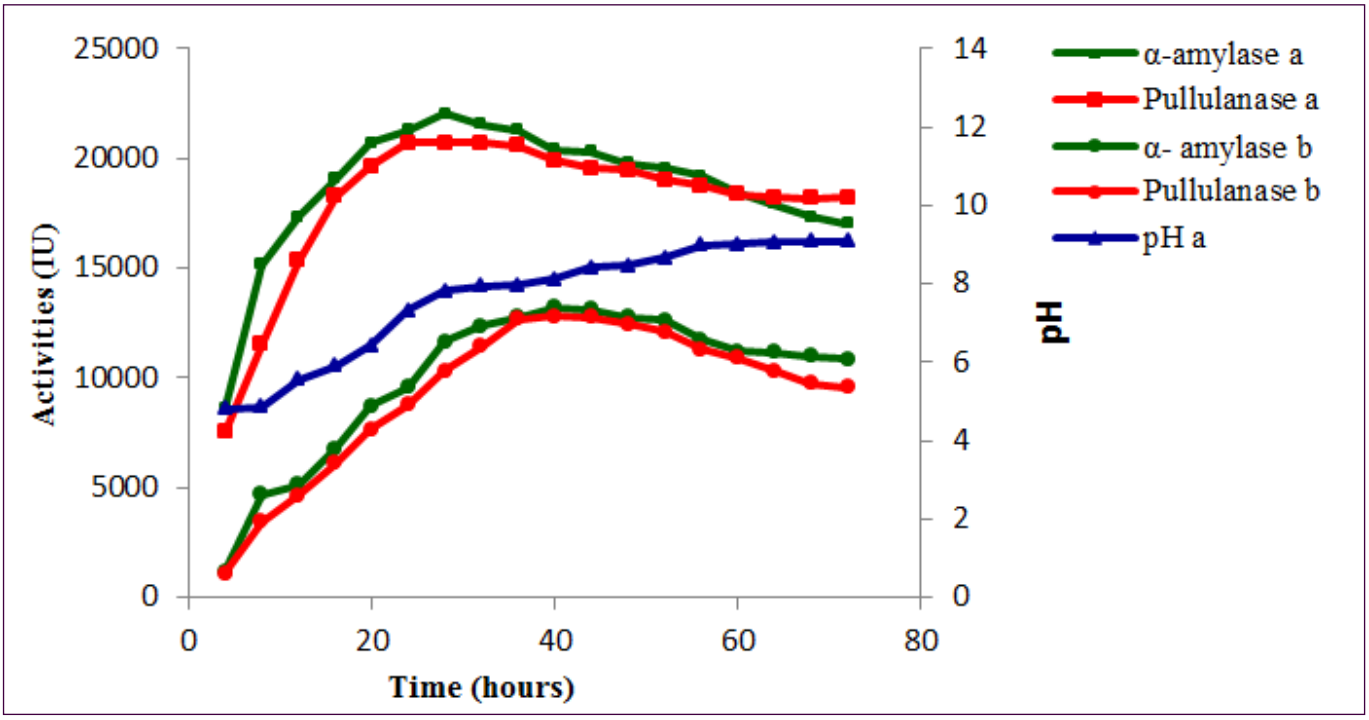

Fig. 3- α- amylase and pullulanase production and, pH profile in optimized medium in fermenter (a) and shake flasks (b).

Conclusion

In this study, obvious enhancement of the amylopullulanase production was obtained. The results indicate that sequential methodology based on the application of the Plackett-Burman and Box-Wilson designs would be a viable and effective alternative for the optimization of the fermentation process. Enzymatic production has doubled in the optimized medium compared to the non-optimized medium.

In addition to the improvement of enzyme production, this study enabled us to ascertain certain characteristics of the new amylolytic *Clavispora lusitaniae* ABS7 strain. The ability of this yeast to produce pullulanase and α-amylase, at 54.14 ° C and pH8, make it possible to obtain a thermostable alkaline alpha amylase. These performances of strain isolated from an aride saharian environment qualify it to be an industrial utility strain.

Conflicts of Interest: None declared.

References

[1] BBC Research (2011) *Enzymes in industrial applications: global markets*, Report Highlights, BIO030F.

[2] Van Der Maarel M.J., Van Der Veen B., Uitdehaag J., Leemhuis H. & Dijkhuizen L. (2002) *Journal of Biotechnology*, 94(2), 137-155.

[3] Gupta R., Gigras P., Mohapatra H., Goswami V.K. & Chauhan B. (2003) *Process Biochem.*, 38, 1599-1616.

[4] Mitidieri S., Martinelli A.H.S., Schrank A. & Vainstein M.H. (2006) *Bioresour. Technol.*, 97(10), 1217-1224.

[5] Hii S.L., Tan J.S., Ling T.C. & Ariff A.B. (2012) *Enzyme Research*, 921362.

[6] Zareian S., Khajeh K., Ranjbar B., Dabirmanesh B., Ghollasi M., & Mollania N. (2010) *Enzyme Microb. Tech.*, 46(2), 57-63.

[7] Marotta M., Martino A., DeRosa A., Farina E., Carteni M. & De Rosa M. (2002) *Proc Biochem.*, 38(1), 101-108.

[8] Gomes I., Gomes J.D. & Steiner W. (2003) *Bioresour. Technol.*, 90, 207-214.

[9] Saxena R.K., Dutt K., Agarwal L. & Nayyar P. (2007) *Bioresour. Technol.*, 98, 260-265.

[10] Lin L.L., Chyau C.C. & Hsu W.H. (1998) *Biotechnol. Appl. Biochem.*, 28, 61-68.

[11] Ouédraogo N., Savadogo A., Zongo C., Somda K.M., Traoré A. S. (2012) *Int. Food Res. J.*, 19(4), 1463-1469.

[12] Moubasher H., Salwa S. & Abou El Kassem W.N. (2010) *Microbiol.*, 6, 759-766.

[13] Plackett R. & Burman L.J.P. (1946) *Biometrika*, 37, 305-325.

[14] Zhang H., Tian Y., Bai Y., Xu X. & Jin Z. (2013) *Int. J. Biol. Macromol.*, 52, 38-44.

[15] Box G.E.P. & Wilson K.B. (1951) *J. Royal Stat. Soc.*, 13(B), 1-45.

[16] Bernfeld P. (1955) *Methods in Enzymology*, Academic Press, New York, 140-146.

[17] Mc Tigue M.A., Kelly C.T., Fogarty W.M.E. & Doyle M. (1994) *Biotechnol. Lett.*, 16(6), 569-574.

[18] Sharma K., Sarita B., Pau S.M., Khurana U. & Kumar K. (2014) *Brit. Microbiol. Res. J.*, 4(4), 369-380.

[19] Irfan M., Nadeem M. & Syed M. (2012) *J. Cell Mol. Biol.*, 10(1), 55-64.

[20] Nair R. & Sabharwal S. (2013) *Res. J. Biotechnol.*, 8(2), 90-95.

[21] De Mot R. & Verachtert H. (1987) *Eur. J. Biochem.*, 164, 643-654.

[22] Antranikian G., Herzberg C. & Gottschalk G. (1987) *Appl. Environ. Microbiol.*, 53(7), 668-1673.

[23] Pedersen H. & Nielsen J. (2000) *Appl. Microbiol. Technol.*, 53 (3), 278-281.

[24] Mrudula S. (2010) *Malays. J. Microbiol.*, 6(2), 181-195.

[25] Asgher M., Asad M.J., Rahman S.U. & Legge R.L. (2007) *J. Food Eng.*, 79, 950-955.

[26] Asha R., Niyonzima F.N. & Sunil S.M. (2013) *Int. Res. J. Biol. Sci.*, 2(3), 35-43.

[27] Boel E., Brady L., Brzozowski A., Derewenda M., Dodson, G. G. & Jenson V.J. (1990) *Biochem.*, 29(26), 6244-6249.

[28] Dean A.C.R. (1972) *J. Appl. Chem. Biotechnol.*, 22, 245-259.

[29] Costanzo B. & Antranikian G. (2002) *Curr. Opin. Chem. Biol.*, 6, 151-160.

[30] Gao H. & Wen-Ying G. (2007) *Biochem. Eng. J.*, 33, 202-210.

[31] Bari M.N., Alam M.Z., Muyibi S.A. & Jamal P. (2009) *Bioresource Technology*, 100(12), 3113-3120.

[32] Narang S. & Satyanarayana T. (2001) *Lett. Appl. Microbiol.*, 32, 31-35.

[33] Humphrey A. (1998) *Biotechnol. Prog.*, 14, 3-7.

[34] Gonzalez C.F., Farina J.I.L. & de Figueroa I.C. (2008) *Enzyme Microb. Technol.*, 42, 272-277.

[35] Clementi F. & Rossi J. (1986) *Antonie van Leeuwenhoek*, 52(4), 343-352.

[36] Namasivayam S.K.R. & Nirmala D. (2011) *Indian Journal of Marine Sciences*, 40(1), 130-133.