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Effect of inorganic nitrogen and phosphorous sources on hydrolyase complex production by a selected *Bacillus subtilis* polar strain

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Abstract

The selected Bacillus subtilis strain, coded MIUG 6.150, isolated from the soil of the East Antarctic coast, generates simultaneously a hydrolyase complex based on the amylase (α-amylase and β-amylase) and protease activity by cultivation in a natural ingredients medium with soy flour, bran, corn steep liquor and inorganic sources of nitrogen and phosphorous (complex manure and $(NH_4)_2HPO_4$). A considerable variability of the biosynthesis potential was found by the quantitative and qualitative variation of nitrogen and phosphorous sources in the liquid fermentative medium by cultivation in submerged conditions. Depending on the specific needs, it is possible to change the ratio of the particular enzymes in the enzymatic complex by changing the mineral composition of the fermentative medium.

Keywords**:** *Bacillus subtilis*, polar soils, hydrolase complex, amylases and proteases biosynthesis

Introduction

The literature provides data on the new microorganisms strains isolated from polar soils, herbarium samples, glaciers and ice-cores; after more than 50 years' preservation they have been successfully revived. Such microorganisms are extremely important for future researches as their scientific implications are quite far reaching. They could have a significant impact on the evolutionary theories. There are also industrial implications to preserve these microorganisms because the new isolates are attractive candidates to screen for new microbial products.

The enzymatic preparation of amylases and proteases complex obtained from *Bacillus* sp. selected strains cover a large range of applications: brewery, bread making, detergents industry or bioremediation [5, 7].

The selection of strains capable of producing simultaneously amylases and proteases featuring increased biosynthesis yields has an obviously practical importance when making commercial enzymatic products for various applications. Thus, depending on the practical necessities, through a proper monitoring of the biosynthesis processes, modification of the enzymes properties in the enzymatic complex is possible [6,8].

The paper presents a simple way of guiding and monitoring biosynthesis with a view to modifying the enzymes proportion by changing the mineral composition of the fermentative medium.

Material and Methods

Microorganisms, media and cultural conditions. The *Bacillus subtilis* MIUG 6.150 strain was isolated, in 1999, from polar soil, the area Larsemann Hills Progress Station, East Antarctic coast. The culture belongs to the Microbiology Laboratory of Galati University (Collection of Microorganims acronym MIUG) and is preserved by adsorption of the spores on sterile sand grains [2].

To establish the influence of the medium composition on the enzymatic complex production we start from a basal medium containing bran, soy flour, corn steep liquor and $CaCl_2$ [3]. A number of nine variants of fermentative media were conceived and examined to quantitatively check the optimal composition of the inorganic sources of nitrogen and phosphorous (complex manure and $(NH_4)_2HPO_4$).

To eliminate other influencing factors use was made of the vegetative inoculum obtained by pure culture cell transfer in a liquid nutrient broth after cultivation to 24-28 hrs on slant agar medium. After 24 hours' cultivation in submerged conditions, 2% cell suspension was used to inoculate the fermentative medium variants. Cultivation for enzymes biosynthesis was performed in submerged system, in Erlenmeyer vessels of 500 ml, with 150 ml liquid medium. Cultivation took place 96 hours, at 32ºC, on a rotary shaker at 200 rot/min.

Evaluation of the hydrolase complex activities. The following enzymatic activities were examined in the liquid culture, after biomass separation at 9000 rot/min, for 10 minutes [1]:

- *α-amylase activity* by using a adapted method (MIUG method) based to a selective distinction of the hydrolysis products in 0.1 N Lugol solution. One α-amylase unit according to this method stands for the amount of enzyme which generates a 0.05 decrease in the optical density, for 1 minute, as measured OD at 610 nm, of the colored iodine-starch colored complex, into a 1% starch solution, at pH 7.0 and 70ºC;
- *β-amylase activity* by Merck method. One β-amylase unit represents the amount of maltose (in mg) release from 1% starch as substrate, by 1 ml enzymatic liquid at 55ºC, pH 6.0, for one minute;
- *global protease activity* by Anson modified method. One Anson unit stands for the amount of enzyme which, under the analytical specified conditions (2 % casein as substrate, pH = 8.0; for 15 min, at 30°C) hydrolyzes the casein at a speed that facilitates release, in one minute, the hydrolysis products soluble in the trichloroacetic acid; this provides coloration equivalent, measured by spectrophotometry at OD 570 nm, to that of one mole of tyrosine, in the presence of the Folin-Ciocâlteau reagent.

Results and discussions

It is well known that bacterial proteases and amylases are extracellular enzymes which synthesis depends on the environment conditions. As inductors, in addition to the substrate, the metabolism end products can be effective too [4].

A repression of the proteases biosynthesis process can be caused by the fast-metabolized carbon sources, which generates the catabolism repression and the presence of amino acids and ammonia.

Another particular specific to the extracellular proteases is the existence of a limited period, at the end of the exponential phase and the beginning of the stationary phase, when biosynthesis is produced. Due to these particulars, the designing of the biotechnology to

produce microbial enzymes is based on the physiology of the producing agent and its behavior depending on the medium composition and the cultivation conditions.

The experiment is carried out to provide information on two independent variables: complex manure concentration and the concentration of $(NH_4)_2HPO_4$ on the biosynthesis yield of the enzymes in the amylase-protease complex (α-amylase, β-amylase and protease). The computation program used for optimization purpose is the Table Curve 3D (Jandel Scientific software).

Starting from the composition of a basic medium found optimum for the biosynthesis of the three enzymes in the enzymatic complex a number of nine variants of culture media was develop by varying + and – of the two independent variables, the concentration of complex manure and concentration of $(NH_4)_2HPO_4$ chosen as factors to study (**Table 1** and **Table 2**).

Table 1. The composition of the basal medium and the level of the independent variable variations

Composition of the basal medium				
Nutritive sources		*Concentrations, g%*		
- Bran		5		
- Soy flour		1,2		
- ***Corn steep liquor***		2,4		
- $CaCl_2 \cdot 6H_2O$		0,2		
Independent variables	*Symbol*	*Levels of variation*		
		-1	**0**	**1**
- Complex manure*, g %	**X**	0,5	0	1,5
- $(NH_4)_2HPO_4$, g%	***Y***	0,5	0	1,5

* 16% N_2, 45 % P_2O_5

Cultivating the selected strain *Bacillus subtilis* MIUG 6.150 on the nine culture media resulted by varying the two independent variables (complex manure and ammonia phosphate) and evaluating the enzymatic potential of the enzymes in the amylase-protease complex, after 96 hours' submerged cultivation the following correlations were obtained:

Table 2. Experimental design of independent variables variation

Variants of fermentative media	**Level of variation for independent variables**		**Concentration of inorganic nitrogen and phosphorus sources, g%**	
	X	**Y**	**Complex manure**	**$(NH_4)_2HPO_4$**
1	+1	-1	1.5	0.5
2	-1	+1	0.5	1.5
3	+1	+1	1.5	1.5
4	-1	-1	0.5	0.5
5	0	+1	0	1.5
6	0	-1	0	0.5
7	+1	0	1.5	0

1. Variation of the α-amylase activity. Correlation of the amylase activity and the variation of the inorganic sources of nitrogen and phosphorous meet the polynomial equation ($z = a + bx + cy + dx^2 + ey^2 + fxy$; were z is the response, i.e. enzymatic activity; UA×ml^{-1} enzyme×min^{-1}) chosen and provides the response surface which varies according to the diagram in **Figure 1**. The assumptions regarding the models consistency were verified through the tests ***t*** and ***F*** both calculated for a significance level of $\alpha = 0.05$ (**Table 3**).

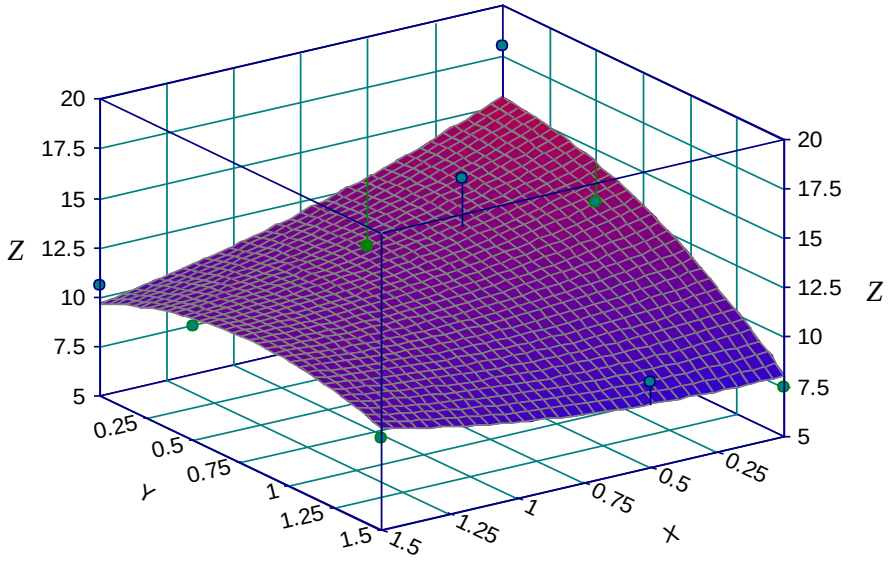

Figure 1. Quantitative and correlative effects of the nitrogen and phosphorous inorganic sources on the bacterial α-amylase biosynthesis

For a mean square deviation equal to $r^2 = 0.6364$ only 63.64% of the experiments fit into the model. From the analysis of the regression equation coefficients the α- amylase biosynthesis yield seems to be strongly influenced by the other components of the fermentative medium (the coefficient takes ***a*** very high value); as regards the variables tested, their absence leads to new max enzyme activities. Also o positive influence on the biosynthesis can be that of the interaction between the two independent variables. Not all the regression coefficients are significant, so in order to establish a real model, only the coefficients falling within the confidence range will be used.

Table 3. Estimation of the correlation coefficients according to the α- amylase biosynthesis model

Regresion coeficients		Std. Erorr	t*	95% Confidence limits		P>|t|	F Statistic	P>F
	Value			min.	max.			
a	15.4131	2.7034	5.7012	6.8733	23.9530	0.0107	1.0506	0.5173
b	-5.4799	7.7337	-0.7085	-29.9095	18.9497	0.5296		
c	-0.8509	7.7337	-0.1100	-25.2805	23.5787	0.9193		
d	1.1073	4.6513	0.2380	-13.5854	15.8000	0.8271		
e	-2.6906	4.6513	-0.5784	-17.3834	12.0020	0.6035		
f	3.4283	2.7683	1.2384	-5.3163	12.1730	0.3036		

* the values of the STUDENT test for the regression coefficients

2. Variation of the β-amylase activity. Under identical analysis conditions, the response surface reflecting the correlation between the quantitative variation of the nitrogen and phosphorous inorganic coefficient in the fermentative medium composition and the β-amylase activity is illustrated in **Fig. 2**. The values of the regression and correlation coefficients, their significance and the confidence intervals are all presented in **Table 4**.

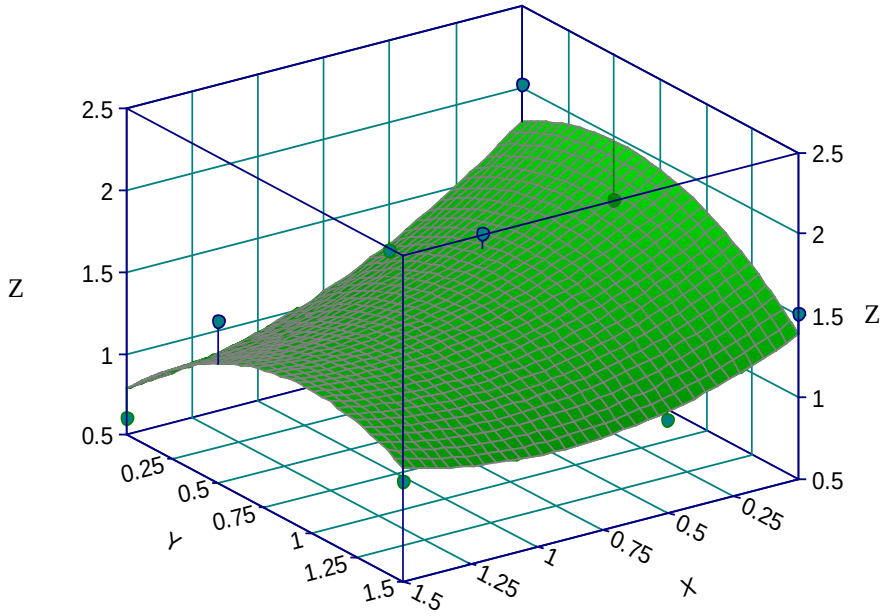

Fig. 2. The effect of the nitrogen and phosphorous inorganic sources on the bacterial β-amylase biosynthesis

The regression equation standing for the variation of the extracellular β-amylase biosynthesis yield depending on the independent variables (complex manure concentration and ammonium phosphate) is valid for 76.64 % of the experiments.

Table 4. Statistical parameters according to the β-amylase biosynthesis model

Regresion coeficients			t*	95% Confidence limits			F	P>F
	Value	Std. Erorr		min.	max.	P>|t|	Statistic	
a	1.7971	0.2719	6.6083	0.9381	2.6562	0.0070		
b	-1.2804	0.7779	-1.6458	-3.7378	1.1770	0.1983	1.95759	0.30787
c	0.6671	0.7779	0.8575	-1.7903	3.1246	0.4541		
d	0.4053	0.4678	0.8662	-1.0726	1.8833	0.4500		
e	-0.6271	0.4678	-1.3402	-2.1051	0.8508	0.2726		
f	0.3626	0.2784	1.3022	-0.5170	1.2422	0.2838		

The following conclusions become obvious from the analysis of the polynomial equation coefficients:

- the max. value of the coefficient ***a*** confirms the existence of other independent variables which influence the β-amylase biosynthesis yield;

- the presence of ammonia phosphate positively influence the β-amylase biosynthesis yield, as confirmed by the relation ***c*** > ***b***;

- the max values of the β-amylase biosynthesis yield are recorded in the absence of the complex manure and at concentrations of 0.5 % ammonium phosphate.

3. Variation of the protease activity. To investigate the effect of the two independent variables on the alkaline protease activity the gross preparation enzymatic activity of hydrolase enzymatic complex was studied. The response surface according to the polynomial equation varied as shown in **Fig. 3**. The polynomial equation chosen meet in 73.98 % experiments to values of the regression and correlation coefficients illustrated in **Table 5**.

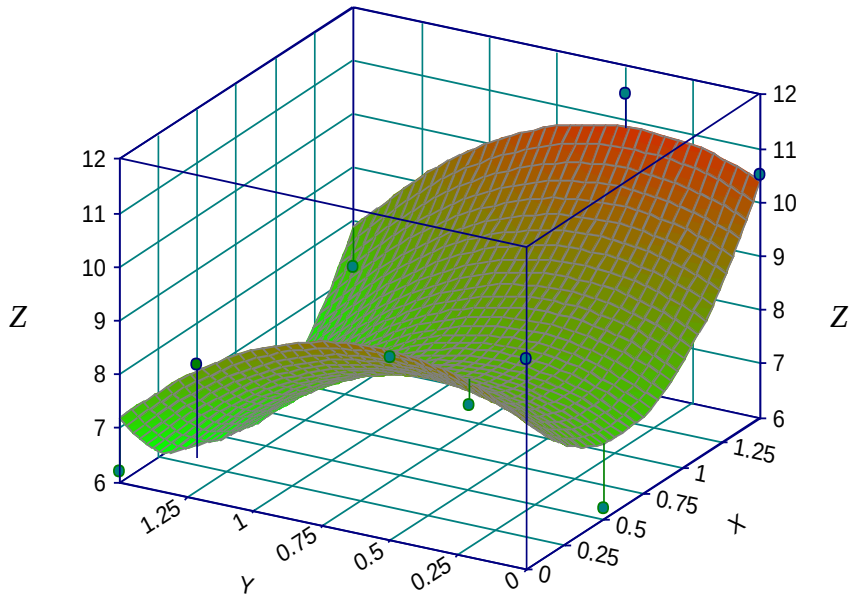

Figure 3. Correlated effect of the nitrogen and phosphate mineral sources on the bacterial protease accumulation

Table 5. Estimation of the correlation coefficients and the confidence intervals for the model of bacterial protease biosynthesis

Regresion coeficients		Std. Erorr	t*	95% Confidence limits		P>|t|	F Statistic	P>F
	Value			min.	max.			
a	8.7834	1.3643	6.4381	4.4738	13.0930	0.0076		
b	-4.6366	3.9028	-1.1881	-16.9648	7.6916	0.3203	1.70638	0.35011
c	2.7866	3.9028	0.7141	-9.5416	15.1149	0.5267		
d	3.8142	2.3473	1.6249	-3.6003	11.2288	0.2026		
e	-2.5696	2.3473	-1.0947	-9.9842	4.8449	0.3536		
f	-0.4068	1.3970	-0.2912	-4.8198	4.0060	0.7898		

From this regression equation too it can be seen the existence of other independent variables which have a considerable effect on the biosynthesis yield (coefficient ***a*** takes a very high value as compared with the other coefficients).

The max protease activity of the gross preparation can be reached with approx.1.5 % complex manure and 0.42 % ammonia phosphate. The ammonia phosphate has influence on the alkaline protease biosynthesis which is also certified by the values $\boldsymbol{c} > \boldsymbol{b}$. The complex manure is a condition for the alkaline protease biosynthesis through a nonlinear correlation its effect being underlined by the high value taken by the coefficient ***d*** which is almost equal to that of coefficient.

CONCLUSIONS

1. According to the response surface methodology the effect of some nutrients as inorganic sources of nitrogen and phosphorous, complex manure and ammonium phosphate on the enzyme biosynthesis yield in the amylase-protease complex obtained with the *Bacillus subtilis* selected strain MIUG 6.150 was studied.
2. The max yield of the α-amylase biosynthesis is reported in the absence of both mineral sources.
3. In the case of β-amylase, max biosynthesis yields are obtained on media without complex manure and 0.5% ammonia phosphate.
4. The biosynthesis of the bacterial protease is significantly affected by the complex manure, max biosynthesis yield being obtained at a concentration of 1.5% in the medium, which is maximum for these experiments. The presence of the ammonia phosphate is favorable in this case, as the concentrations of 0.42 % together with 1.5 % complex manure makes possible for max protease yield to be achieved.
5. The study ensures the control and handling of the enzyme biosynthesis in the amylase-protease complex by using a base medium of natural ingredients and stimulating the biosynthesis of the complex enzymes using mineral sources of nitrogen and phosphorous.
6. The research has demonstrated that biosynthesis of the three enzymes is also influenced by other components in the fermentative medium which effect needs further studies and optimization.

Acknowledgements
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References

1. G. BAHRIM, A. NICOLAU, *Biotehnologia preparatelor enzimatice. Tehnici si analize de laborator*. Ed. Academica Galati (2002)
2. G. BAHRIM, T. NEGOIȚĂ, *3rd Annual Meeting of Polar Section of the Czech Geographical Society ACADEMIC AND UNIVERSITY CENTRUM* - Nove Hrady near Trebon, Czech Republic (2002)
3. V. DAN, G. BAHRIM, A. NICOLAU, Roumanian Patent No 14150 (1999)
4. R. MULLER, *Enzyme and Microbial Technology,* **27**, 337–344 (2000)
5. T. GODFREY, J. REICHELT, *Industrial Enzymology the Application of Enzymes in Industry*. Nature Press. New York (1983)
6. F.G. HEINEKEN, F.G. OCONNER, *J. Gen Microbiol,* **73**, 35 (1972)

7. F.G. PRIEST, *Products and Applications In: Bacillus Biotechnology*. Hand Book 2, Plenum. New York-London (1989)
8. Y. YONEDA, B. MARVA, *J. Bacteriol*., **124**, 48-54 (1975)