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Optimization of extracellular catalase production from *Aspergillus phoenicis K30* by a linear regression method using date flour as single carbon source and purification of the enzyme

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***Aspergillus phoenicis* K30 is the selected mutant which produces an amount of extracellular catalase. To amplify the extracellular catalase production by the strain, a fermentation optimization was performed. To select the factors affecting the production, nine active variables (factors) consisting of 12 experiments were analyzed by Plackett-Burman design. Each variable was tested at two levels, a higher and a lower level. The studies of the effect of each variable and the establishment of a correlation between the response of enzyme activity and variables revealed that the link is a multiple linear regression form. The optimization was carried out through a simplex algorithm. The amount of extracellular catalase produced by the strain in the optimized medium was about four times higher than that obtained in non optimized medium corresponding to 3820 mg/L of extracellular proteins including 59500 U/L of extracellular catalase activity after 96 h of fermentation. The steps of purification were allowed to improve enzyme activity by 305-fold. From an analytical gel electrophoresis under native conditions, an apparent molecular mass of 158 kDa was determined suggesting that the enzyme is a homodimer. The isoelectric point of the protein was found to be 5 ± 0.1 as determined by a Pharmacia Phast-system.**

Key words: *Aspergillus phoenicis*, extracellular catalase purification, dates flour, optimization, multiple linear regression.

INTRODUCTION

Catalase (H_2O_2 oxidoreductase, EC.1.11.1.6) is an enzyme that catalyses the decomposition of hydrogen peroxide to oxygen and water. Catalase is widely distributed among life forms and found in all aerobic microorganisms, which have evolved a specific enzyme system to neutralise potentially lethal effects of hydrogen peroxide (Halliwell, 1990, Zamocky et al., 2008). Catalase is used in several industrial applications such as food or textile

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processing to remove hydrogen peroxide that is used for sterilisation or bleaching (Akertek and Tarhan, 1995). This enzyme is located in the cytoplasm or in peroxisomes but not usually excreted from the cell. Industrially, the extracellular liberation of an enzyme is more advantageous than extracting the intracellular one. Thus, a cheap and readily available commercial source of catalase can be found in the extracellular production by several microorganisms, especially fungi strains. A few studies reported the influence of environmental conditions on the extracellular catalase production (Gromada and Fiedurek, 1997; Fiedurek and Gromada, 1997; Isobe et al., 2006). However, it has been shown that an amount of extracellular catalase could be produced naturally by microscopic mycelial and unicellular fungi in synthetic media (Kurakov et al., 2001; Mikhailova et al., 2001) and in the environ-ment of fungus infected barley roots (Zhang et al., 2004). Recently, the production of extracellular catalase was noted in different investigations of the defense evolved by several strains of fungi attacking the plants to eliminate the lethal effect of H_2O_2 secreted by the host (Macarisin et al., 2007; Blackman and Hardham, 2008; Brown et al., 2008).

The catalase production cost can be reduced by using a shipper carbon source and by optimizing the process of production. *Aspergillus phoenicis* K30 is the selected mutant which produces an amount of extracellular catalase (Kacem et al., 2004, 2005). However, to our knowledge, there has been no report of this production on date flour as natural media. Therefore, in the present study, fermentation optimization of this strain was performed to amplify the extracellular catalase production using date flour as single carbon source. Nine active variables were analyzed and optimized by a multiple linear regression.

MATERIALS AND METHODS

Strain

The wild-type strain was isolated from foodstuff, in the East of Algeria and was identified as *A. phoenicis* (Kacem et al., 2004). It was maintained as a spore suspension in 30% glycerol physiological water at -20°C. This strain produces a small amount of an extracellular catalase in solid medium and in shaken submerged culture. The *A. phoenicis K30* is the mutant of the wild-type strain, showing an increase of extracellular catalase (Kacem et al., 2005). The cultures were carried out in 500 ml Erlenmeyer flasks filled with 100 ml medium. The sterilised media were inoculated with a final concentration of 1 x 10^6 spores/ml. The conidia were harvested from seven days old Potato dextrose agar culture (formula in g/L; dextrose 20, microbiological agar 15, infusion from potatoes [200 g] 4, final pH 5.6). The flasks were incubated for 96 h under the culture defined by statistical design (see experimental design).

At the end of the incubation period, the fungal mycelium was separated from the culture fluid by filtration on Whatman paper no. 2. The filtrate was used for determination of extracellular catalase activity and the mycelium was used for dry weight determination.

Experimental design

The first objective of the present experiments was to determine which of the factors listed above were of decisive importance and to quantify their effect. The factors were associated to a response function Y represented by a polynomial model of first order. Then, an optimal response was obtained by applying the simplex method.

The optimization of the fermentation medium for a maximum production of extracellular catalase is based on a statistical plan that takes into account environmental factors in which it evolves mutant *A. phoenicis* K30 (nutritious components of the environment and conditions of incubation) (Kacem et al., 2005). The method of Plakett and Burman (1946) was applied for a quick selection of the most influential factors on one answer (extra cellular catalase production). Indeed, nine variables were tested at two levels [high level HL (+), and low level LL (-)] using 12 experiments (Table 1).

The factors which are supposed to optimize the fermentation medium were selected from the literature. However, the source of carbon is a flour of decommissioned dates (to our knowledge this substance has never been used as carbon source) and the source of nitrogen is a waste of starch "corn steep." The organization of experiments is performed according to Plackett-Burman matrix *(*Plackett and Burman, 1946; Jacques et al., 1999) which is composed of 12 experiments (Table 1). Each column represents the different variables (factors) and each row represents the different experiments. Each factor is tested at two levels, a higher one (+) and a lower one (-).

Data analysis

The main effect *βi* of the variable *i* is the shift in the response (*Yj*), when the corresponding variable coded *Xi* increases by half. Its estimate is given by the equation:

$$ß_i = [\sum_{1}^{n} (\text{signe})_{ij} Y_j]/(n/2) \quad (1)$$

Where, *n* is the number of experiments, and *k* is the number of variables.

i = 1,….*k,* and *j* = 1,…*n.*

The calculation of the effect of each variable and the establishment of a correlation between the response of enzyme activity (*AE*) and the variables *Xi* have been obtained by means of minitab 2000 software. The link is a multiple linear regression form:

$$Y=C_0+C_1*X_1+C_2*X_2+C_3*X_3+C_4*X_4+C_5*X_5 \quad (2)$$

The optimization was carried out through a simplex algorithm (Zaatri 2002) on the objective function:

$$F=C_1*X_1+C_2*X_2+C_3*X_3+C_4*X_4+C_5*X_5 \quad (3)$$

$$F = Y - C_0 \quad (4)$$

Where, *Y* is the function to maximize [enzyme activity (*EA*)]; *Xi* is the variables to optimize

The following data were taken into account: the number of variables (*n*), the number of constraints (*m*), the diagonal matrix of coefficients a (*i, j*), where: *i* = 1,… *k* and *j* = 1,… *k* ; the vector *B* (bi) of constraints and the vector *C* (*cj*) of cost coefficients (*C1, C2, C3,……., Cj*).

Purification procedures

Crude enzyme solution

After 96 h of fermentation, the culture was filtered. The filtrate

Table 1. The matrix of Plackett-Burman (1946) composed of 12 experiments at two levels and responses.

Variable		T (°C)	pH	Shaking speed (rpm)	Dates flour	Corn steep[a]	$NaNO_3$[a]	KH_2PO_4[a]	$MgSO_4$[a]	$FeSO_4$[b]	Response	
LL (-)		24	4.0	140	40	5	2	0.5	0.25	10	CDW (g/L)	EA (U/mL)
HL (+)		30	6.0	200	80	10	4	1.5	0.75	40		
Experiment	1	+	+	-	+	-	-	+	-	+	10.52	11.50
	2	+	-	+	-	-	-	+	+	+	26.04	20.00
	3	-	+	+	-	+	-	+	-	-	17.72	17.50
	4	+	+	+	-	-	+	-	+	-	15.30	**48.25**
	5	+	+	-	+	+	-	-	+	-	19.94	14.25
	6	+	-	-	-	+	+	-	-	+	14.41	7.50
	7	-	-	-	+	-	+	+	+	-	25.16	4.50
	8	-	-	+	+	+	-	-	+	+	**29.95**	15.00
	9	-	+	-	-	+	+	+	+	+	18.65	12.50
	10	+	-	+	+	+	+	+	-	-	26.53	2.50
	11	-	+	+	+	-	+	-	-	+	27.21	17.50
	12	-	-	-	-	-	-	-	-	-	16.72	1.00

[a]Concentration (g/L) ; [b]Concentration (mg/L); CDW, cell dry weight ; E.A, extracellular catalase activity ; LL, low level; HL, high level.

was used for the purification of the extracellular catalase, in which, the initial activity was estimated at 59.5 U/ml as described (see analytical methods). The solution was dialysed under stirring (4°C) in 500 ml distilled water (three changes of distilled water) then, the solution was dialysed against 20 mM-phosphate buffer pH 7.

DEAE-Sepharose CL-6B ion exchange chromatography

32 ml of the dialysed solution were applied with a loading rate of 60 ml/h to a column XK (1 x 12 cm) (Pharmacia, Sweden) containing DEAE-Sepharose CL-6B previously conditioned with 20 mM phosphate buffer pH 7 and connected to a chromatographic system (Bio-Rad, U.S.A.). The column was then thoroughly washed with the starting buffer until the absorbance reached zero at 280 nm. The proteins were eluted (4°C) by a linear gradient of sodium phosphate buffer A (20 mM) and B (500 mM) pH 7, at a flow rate of 1.2 ml/min. The gradient was from 100% A to 100 % B, for 90 min. The fractions of 1 ml were collected and the activity of catalase was measured in each tube. Eight fractions showed an increase of catalase activity better than 10% of initial activity.

Ultrafiltration

The active fractions were mixed and concentrated by ultrafiltration which was performed at 4°C and 3 bars N_2 using a Centriprep-10 Model 8400 membrane with a molecular weight cut-off of 10 kDa (Amicon, U.S.A.).

Electrophoresis

Native PAGE electrophoresis was used to determine the purity of fractions and the molecular weight of the purified enzyme. Pharmacia PhastSystem was used for non-denaturing PAGE (12% polyacrylamide, Multi-Cell chamber Bio-Rad, USA). The molecular weight standard (high range, Bio-Rad, USA) was myosin (200.0 kDa), ß-galagtosidase (116.3 kDa), phosphorylase (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45,0 kDa) and carbonic anhydrase (31,0 kDa). 10 µl of the mixture were applied in each well. The electrophoresis was done at 4°C for 45 min at constant voltage (200 V) and the elution buffer was glycine 7.5% and Tris-base 1.5%, pH 7 (Park et al., 2000). Protein bands was stained by Coomassie Brilliant Blue (0.025 %) (Krueger et al., 1977).

Isoelectric focusing

The isoelectric focusing experiments were performed with the Pharmacia PhastSystem using the conditions recommended by the manufacturer. Polyacrylamide gel (Ampholine PAGE-plate pH 4 to 7) and low IEF pH, 2.5 to 6.5, covering the appropriate range and the broad calibration kit were used for isoelectric focusing (Leoni et al., 1998).

Analytical methods

All protein estimations were made using the bicinchoninic acid assay according to Sigma procedure No, TPRO-582. Protein concentrations in samples were estimated from the absorption at 280 nm. The catalase activity was measured spectrophotometrically (Pharmacia, mod.UV), by observing the decrease in light absorption at 240 nm during decomposition of H_2O_2 by the enzyme. The reaction mixture (3 ml) contained 0.1 M phosphate buffer pH 7.5, 0.03 ml of a suitably diluted enzyme and 0.5 ml of 108.8

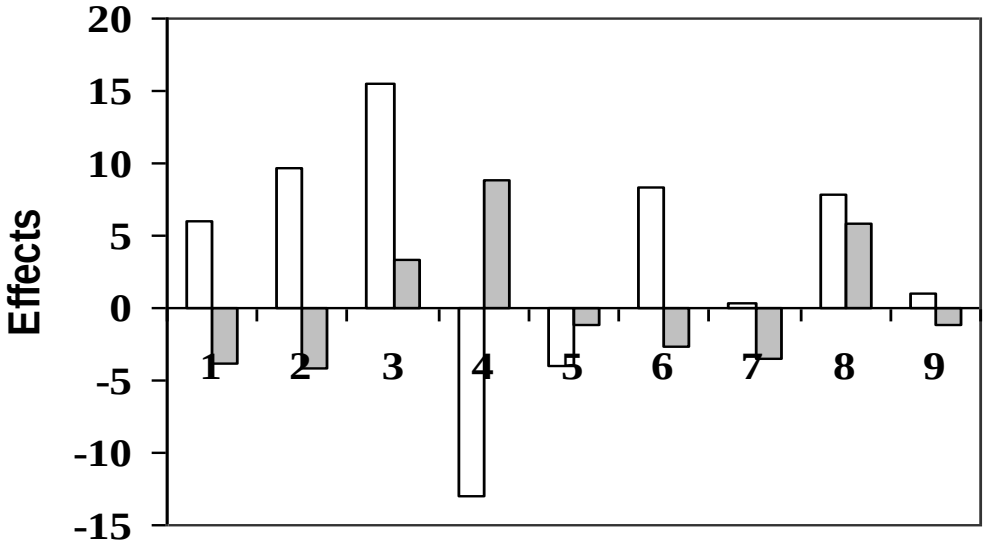

Figure 1. Effects of different factors on extracellular catalase activity (ϒ) and on the biomass (■): 1, Temperature; 2, pH; 3, shaken speed; 4, dates flour; 5, corn steep; 6, Na NO_3; 7, KH_2PO_4; 8, $MgSO_4$; 9, $FeSO_4$.

mM H2O2 solution. One unit (U) of catalase activity was defined as the amount of enzyme catalyzing the decomposition of one µmol of H2O2 min-1 at 25°C and pH 7.5 (Beers and Sizer, 1952; Caridis et al., 1991).

RESULTS

For rapid identification of the main variables affecting the extracellular catalase production by *A. phoenicis* K30, using the soluble extract dates flour as single carbon source, a Placket-and Burman design of 12 experiments were carried out with two levels. The first goal was to select the variables (factors) and second the mathematical regression was used for modelling. The variables and their levels were selected from literature and are shown in Table 1.

This optimization allowed us to define new cultural conditions adapted for high extracellular catalase production. The tested factors are reflected in the Table 1 where biomass ranged from 10.5 (medium 1) to 30 g/L (medium 7). As for the extracellular catalase activity, this was used from 1 (medium 12) to 48 U/mL (medium 3).

The model gave a high coefficient of determination (R^2) and a low probability ($P < 0.05$) was subjected to a thorough optimization (maximization of responses) by the simplex method. Each of the factors had a significant and positive effect on the production of extracellular catalase; shaking speed (15.417), pH (9.6), temperature (6.017), concentration of $NaNO_3$ (8.333) and $MgSO_4$ (7.883). In addition, a significant negative effect was noted for flour dates (-13.000) and to a lesser degree for the "corn steep" (-4.000). Other factors: KH_2PO_4 and $FeSO_4$ had no effect on enzyme activity (Figure 1). The production of the enzyme by *A. phoenicis* K30 was thus, improved by increasing the shaking speed, pH, temperature and $MgSO_4$ and a decrease in the concentration of date flour.

Also, the factors that have a significant effect on the yield of biomass were studied (results not discussed here) but apparently, the factors influencing biomass are different from those affecting the production of the enzyme.

To confirm the previous selection, a modelling study based on the establishment of a multiple linear regression linking the different factors (X_i) to the responses (Y_j) was undertaken by next logical mathematics:

$$Y = C_0+C_1X_1+C_2X_2+C_3X_3+\ldots+ C_kX_k$$

Where Y is the responses, $X_1,\ldots.X_0$ are variables; $C_0,\ldots C_k$ are coefficients of correlation and k is the number of factors.

Indeed, the modelling of enzyme activity based on the studied factors resulted in a multiple regression equation linking; the temperature, the pH, the shaking speed, the concentration of dates flour, the corn steep, the $MgSO_4$ with a coefficient of determination significant ($R^2 > 95$ %) and a probability of 4.5%. So, this model is the most accepted for enzyme activity correlate to the 6 factors.

Indeed, the enzyme activity was correlated with the temperature, pH, shaking speed, concentration of dates flour, corn steep and $MgSO_4$ using the following equation:

Table 2. Purification of extracellular catalase of *A. phoenicis* K30 grown on date flour soluble extract submerged medium using a DEAE-Sepharose CL-6B ion exchange chromatography.

Purification step	Proteins (mg/ml)	Total proteins (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	3.82	122.2	59.5	1904	15.6	100	1.00
Dialysed extract	3.72	119.2	57.0	1824	15.3	98.3	0.98
After DEAE-Sepharose CL-6B ion exchange chromatography	0.04	0.35	212.4	1700	4761.9	89.3	305.8

$Y = -62.91 + 1*X_1 + 5.93*X_2 + 0.193*X_3 - 0.173*X_4 - 1.12*X_5 + 19*X_6$

Where, X_1 is the temperature, X_2 is the pH, X_3 is the shaking speed, X_4 is the dates flour, X_5 is the corn steep and X_6 is $MgSO_4$. The KH_2PO_4 and the $FeSO_4$ effects were not significant, so they were not selected in the determination of this correlation. This model confirms the significance of the effects of factors selected by the matrix Plakett-Burman except for the factor $NaNO_3$.

The logic of simplex was applied to optimize enzyme activity (extracellular catalase) based on the regression equation with the selected variables:

The objective function was:

$F= 1*X_1 + 5.93*X_2 + 0.193*X_3 - 0.173*X_4 - 1.12*X_5 + 19*X_6$

The execution of the simplex algorithm was based on the following constraints and modified variables:

$24 \leq X_1 \leq 30$

$4 \leq X_2 \leq 6$

$140 \leq X_3 \leq 200$

$40 \leq X_4 \leq 80$

$5 \leq X_5 \leq 10$

$0.25 \leq X_6 \leq 0.75$

Modified variables were:

$y_1 = X_1 - 24$ $\quad 0 \leq y_1 \leq 6$
$y_2 = X_2 - 4$ $\quad 0 \leq y_2 \leq 2$
$y_3 = X_3 - 140$ $\quad 0 \leq y_3 \leq 60$
$y_4 = X_4 - 40$ $\quad 0 \leq y_4 \leq 40$
$y_5 = X_5 - 5$ $\quad 0 \leq y_5 \leq 5$
$y_6 = X_6 - 0.25$ $\quad 0 \leq y_6 \leq 0.5$

The function Y is written as:

$Y = 4.06 + 1*y_1 + 5.93*y_2 + 0.193*y_3 - 0.173*y_4 - 1.12*y_5 + 19*y_6$

The data for carrying out the simplex algorithm are:

Vector B (bi): (6, 2, 60, 40, 5, 0.5)

Vector C (cj): (1, 5.93, 0,193, -0,173, -1.12, 19).

The result obtained after several iterations was: max F = 38.94 or $Y_{max} = 43.00$
With: $X_1 = 30$, $X_2 = 6$, $X_3 = 200$, $X_4 = 40$, $X_5 = 5$, X6 =0.75

For a maximal rate of extracellular catalase activity, the variables (factors) must be fixed to: T = 30 °C (factor X_1), pH = 6 (factor X_2), speed of agitation = 200 rpm (factor X_3), flour dates = 40 (g/l) (factor X_4), « corn steep » = 5 (g/l) (factor X_5), $MgSO_4$ = 0.75 g/l (factor (X_6)

Purification of enzyme

After 96 h of culture in optimized medium including DFSE as a carbon source, *A. phoenicis* K30 produced 3820 mg/l of extracellular proteins including 59500 U/l of extracellular catalase activity. Table 2 shows the purification steps for extracellular catalase starting from a filtrate of fermentation culture. This purification makes it possible to obtain a final specific activity of extracellular catalase of 4761.90 U/mg improving enzyme yield by 305-fold. Indeed, Figure 2 shows that the fractions which showed a catalase activity were eluted when the concentration of buffer began to increase. Thus, the fractions containing activity better than 10% of initial activity (8 fractions) were collected. The native PAGE electrophoresis applied to determinate the purity of fractions, point out that all fractions presented a single band. The fractions include a total protein of 0.352 mg and amount of a total extracellular activity estimated at 1700 U.

From an analytical gel electrophoresis under native conditions, an apparent molecular mass of 158 kDa was determined suggesting that the enzyme is a homodimer (Figure 4).

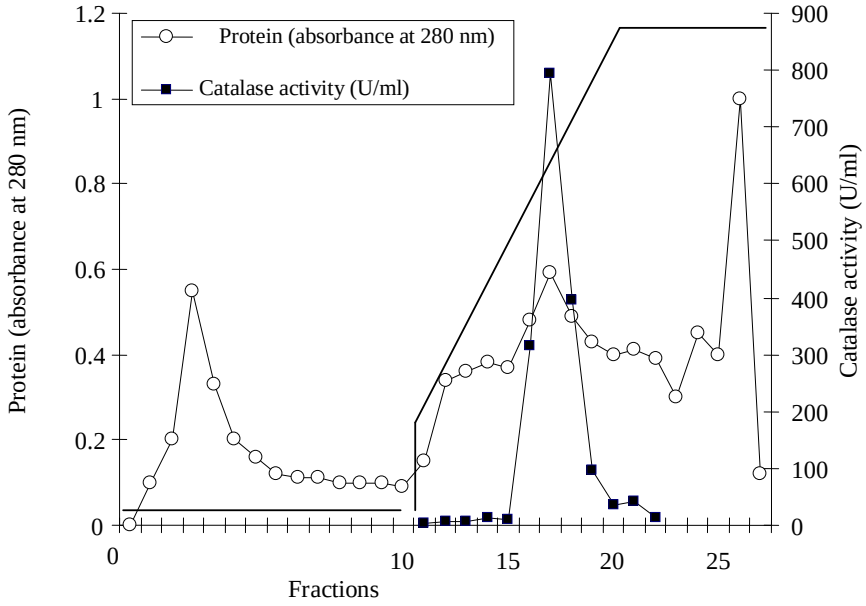

Figure 2. Chromatography of extracellular catalase on DEAE-Sepharose CL 6B. (-o-) Protein; (-■-) extracellular catalase activity; (-) a linear gradient of sodium phosphate buffer using (20 to 500 mM, pH 7).

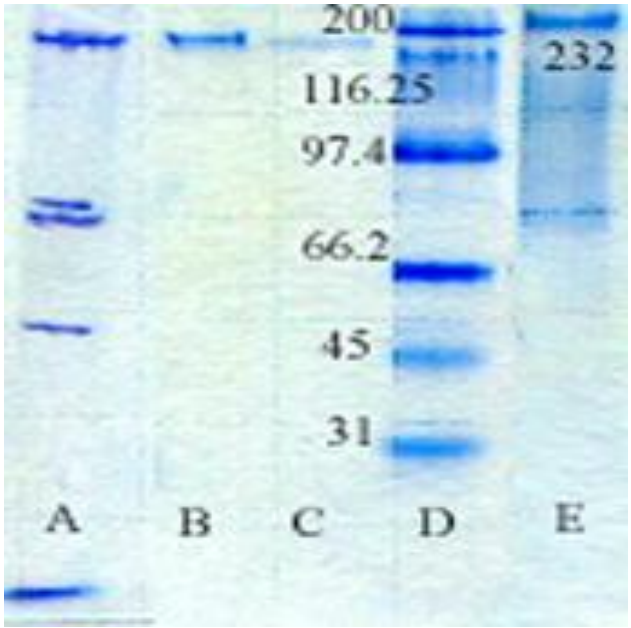

Figure 4. Native poly acrylamide gel electrophoresis; (A) crude enzyme, (B, C) purified extracelluar catalase, (D) standard protein, (E) Fluka catalase.

The confirmation of the exact molecular weight is achieved by HPLC with a column of type Progel, TSK G3000 (Supelcs). The results show that the catalase has a retention time of 11,736 min, situated between that of myosin (200 kDa) of 10,817 min and that of the beta-galactosidase (116 kDa) of 12,556 min.

The isoelectric point of the protein was found to be 5 ± 0.1 as determined with a Pharmacia Phast-system.

DISCUSSION

The estimated effects of each factor and the selection of the most significant factors correlated with the response to selected factors in the form of a multiple linear regression. The latter has been optimized for maximization of the response, using the simplex method (Zaatri, 2002). In addition, the study showed that biomass and enzymatic activity are not influenced by the same factors and, consequently, the production of extracellular catalase by *A. phoenicis* K30 is partly correlated with biomass.

About 50% of all enzymes investigated so far exist in multiple molecular forms, isoenzymes (Scandalios, 1968). These usually differ in electrophoretic mobility. Besides, they may have slightly different catalytic abilities. Differences in electrophoretic mobility may result in charge and/or size variabilities. Enzyme multiplicity can directly or indirectly depend on genetic factors. The catalase (alloenzyme) which evolve by post-translational modifications of a given protein structure are named secondary isozyme (Markert, 1977). In mouse kidney, five major forms of catalase enzymes have been found in the soluble fractions. The whole encoded in a single genetic locus and their multiplicity is caused by the progressive attachment of negatively charged sialic acid residues to each of its four subunits (Jones and Masters, 1972; Prakash et al., 2002). The ion exchange column chromatography on DEAE-Sepharose CL 6B appears to be an efficient technique. Unlike the molecular weight, it is due to post-translational modifications, particularly as a result of glycosylation of catalase (Markert, 1977). The electrophoretic profile in non-denaturing conditions (Native-PAGE) of the extracellular catalase produced by *A. phoenicis* K30 indicates a single protein band of low mobility, reflecting a single molecular form (no isoenzymes), high molecular weight or an enzyme in aggregate form. However, the catalase may have several isoenzymes as well, 5 major forms of catalase were determined in the kidney cells of rats. Isoenzymes, however, are coded by the same ancestral genes (Moss, 1982; Gruan and Scandalios, 1996). Moreover, it has been demonstrated that the fungus *Histoplasma capsulatum* produces an extracellular catalase termed M antigen, which is similar to catalase B of *Aspergillus* and *Emericella* species. Evidence is presented here for two additional catalase isozymes in *H. Capsulatum* (Johnson et al., 2002). Catalase A is highly similar to a large-subunit catalase in *Aspergillus* and *Emericella* species, while catalase P is a small-subunit catalase protein with greatest similarity to known peroxisomal catalases of animals and *Saccharomycotina* yeasts (Johnson et al., 2002). The protocol established for the purification of extracellular catalase (crude extract) secreted by the mutant *A. phoenicis* K30 is simple. It led to the purification of this enzyme in one step and without going through the conventional precipitation by ammonium sulphate.

The application of the technique of ion exchange chromatography to purify the enzyme is motivated by its simplicity and its widespread use in many researches for the purification of various proteins (Tsai et al., 1992; Leoni et al., 1998). By the same process, the extracellular catalase of *A. niger* was purified with an enrichment rate of 174 (Rogalski et al., 1998) and the purification of catalase of pseudomonad EF group 70B was achieved with a rate of 151.8 (Kuusk et al., 2001). By the same technique, a single band of active catalase of *Rhodobacter sphaeroides* 2.4.1 ATH was showed and purified with a rate of 304 (Terzenbach and Blaut, 1998), value very close to that obtained in this work (306). The elution of the enzyme with a salt concentration of 400 mM can be explained by the fact that it contains a number of negative charges (carboxylic groups). This observation is supported by the value of low isoelectric point (4.9) determined by electrofocalisation.

The molecular weight of most studied catalases varies between 220 and 270 kDa *(*Furuta et al., 1974; Kirkman and Gaetani, 1984; Hyoung-pyo et al., 1994). The molecular weight of the intracellular catalase of *A. niger* was determined which varies from 323 to 396 kDa (Rogalski et al., 1998). Recently, the existence of several forms of catalase with a lower molecular weight as compared to the previous values, was proven. Indeed, the work concerning the catalase of *Penicillium simplicissimum* and the *Synechococcus* revealed respective molecular weight of 170 kDa and 150 kD (Fraaije et al., 1996; Mutsuda et al., 1996). More recently, the catalase bromoperoxidase of pseudomonad was purified and consists of two subunits having a molecular weight of 153 kD (Kuusk et al., 2001). Moreover, three isoenzymes of the catalase were identified from *Aspergillus fumigatus*, one of them is a dimer with molecular mass of 84.5 kDa (Paris et al., 2003).

In conclusion, the molecular weight of 158 kDa obtained by electrophoresis (in non-denaturing conditions) for the extracellular catalase, produced by *A. phoenicis* K30, can be justified.

REFERENCES

Akertek D, Tarhan L (1995). Characteristics of immobilised catalases and their applications in pasteurisation of milk. Appl. Biochem. Biotechnol. 50: 9555-9560.

Beers RF, Sizer IW (1952). A spectrophotometric methode for measuring the breakdown of hydrogenperoxide by catalase. J. Biol. Chem. 195: 276-287.

Blackman LM, Hardham AD (2008). Regulation of catalase activity and gene expression during *Phytophthora nicotianae* development and infection of tobacco. Mol. Plant Pathol. 9 (4): 495-510.

Brown SH, Oded Y, Gollop N, Chen S, Zveibil A, Belausove E, Freeman S (2008). Differential protein expression in *Colletotrichum acutatum*: changes associated with reactive oxygen species and nitrogen starvation implicated in pathogenicity on strawberry. Mol. Plant Pathol. 9 (2): 171-190.

Caridis KA, Christakopulos P, Macris BJ (1991). Simultaneous production of glucose oxidase and catalase by *Alternaria alternate*.

Appl. Microb. Biotechnol. 34: 794-797.
Fiedurek J, Gromada A (1997). Selection of biochemical mutants of *Aspergillus niger* with enhanced catalase production. Appl. Microbiol. Biotechnol. 47: 313-316.
Fraaije MW, Roubroeks HP, Hagen WR, vanBerkel WJH (1996). Purification and characterisation of an intracellular catalase-peroxidase from *Penicillium simplicissimum*. Europ. J. Biochem. 235: 192-198.
Furuta H, Hachimori A, Ohta Y, Samejima T (1974). Dissociation of bovine liver catalase into subunits on acetylation. J. Biochem. (Tokyo). 76: 481-490.
Gromada A, Fiedurek J (1997). Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase production. J. Appl. Microbiol. 82: 648-652.
Gruan L, Scandalios JC (1996).Molecular evolution of maize catalases and their relationship to other eukaryotic and prokaryotic catalases. J. Mol. Evol. 42(5): 570-579.
Halliwell B (1990). How to characterise a biological antioxidant. Free Radicals Res. Commun. 9: 1-32.
Hyoung-pyo K, Youngchil H, Jung-hye R (1994). Characterisation of major catalase from *Streptomyces coelicolor* ATCC 10147. Microbiol. 140: 3391-3397.
Isobe K, Inoue N, Takamatsu Y, Kamada K, Wakao N (2006). Production of Catalase by Fungi Growing at Low pH and High Temperature. J. Biosci. Bioeng. 101(1): 73-76.
Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, de Pauw E, Thonart Ph (1999). Optimisation of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. Appl. Biochem. Biotechnol. 77-79: 223-233.
Johnson CH, Klotz MG, York JL, Kruft V, McEwen JE (2002). Redundancy, phylogeny and differential expression of *Histoplasma capsulatum* catalases. Microbiology 148:1129-1142.
Jones GL, Masters CL (1972). A survey on the formation and localisation of secondary isosyme on the differential inhibition of the multiple forms of catalase in mouse tissue. FEBS Lett. 21: 207-210.
Kacem- chaouche N, Meraihi Z, Destain J and Thonart Ph (2004). 26^{The} Symposium on Biotechnology for fuels and Chemicals, Session Enzyme Catalysis Technology, May 9-12. Chattanooga, TN USA.
Kacem Chaouche N, Meraihi Z, Destain J, Thonart Ph (2005). Study of catalase production by an *Aspergillus phoenicis* mutant strain in date flour extract submerged cultures. Biotechnol. Agron. Soc. Environ. 9 (3): 173-178.
Kirkman H, Gaetani G (1984). Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. Proc. Natl. Acad. Sci. U S A. 81: 4243-4251.
Krueger BK, Forn J, Greengard P (1977). Depolarization-induced phosphorylation of specific protein, mediated by calcium ion influx, in rat brain synaptosomes. J. Biol. Chem. 252: 2764-2773.
Kurakov AV, Kupletskaya MB, Skrynnikova EV, Somova NG (2001). Search for micromycetes producing extracellular catalase and study of conditions of catalase synthesis. Appl. Biochem. Microbiol. 37 (1): 59-64.
Kuusk H, Björklund M, Rydstöm J (2001). Purification and characterisation of a novel promoperoxidas-catalase isolated from bacteria found in recycled pulp white water. Enzyme Microbiol. Technol. 28: 617-624.
Leoni O, Iori R, Haddoum T, Marlier M, Wathelet JP, Rollin P, Palmieri S (1998). Approach to the use of immobilised sulfatase for analytical purposes and for the production of desulfo-glucosinolates. Ind. Crop. Prod. 7:335-343.
Macarisin D, Cohen L, Eick A, Rafael G, Belausov E, Wisniewski M, Droby S (2007). *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Physiopathology 97 (11): 1491-1500.
Markert CL (1977). Isozymes: the development of a concept and its application. In: Rattazzi MC, Scandalios JG, Whitt GS (edn) Isozymes: Current tropics in biological and medical research, vol. 1. Alan R Liss, New York. pp. 1-16.
Mikhailova RV, Osoka OM, Lobanok AG (2001). Extracellular catalase synthesis by Penicillium fungi. Mycol. Phytopathol. 1 (35):43-47.
Moss DW (1982). Isoenzymes. (edn) Chapman and Hall, London.
Mutsuda M, Ishikawa T, Takeda T, Shigeoka S (1996). The catalase-peroxidase of Synechococcus PCC 7942: purification, nucleotide sequence analysis and expression in *E. coli.* Biochem. J. 316: 251-257.
Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, Diamond RD, Latgé JP (2003). Catalase of *Aspergillus fumigates*. Infect. Immun. 76 (6): 3551-3562.
Park IH, Han BK, Baek JH, Ryu YW, Park YH and Jo DH (2000). Subunits of neurosteroid sulfatase from bovine brain. J. Steroid. Biochem. Mol. Biol. 73 (3-4): 135-139.
Plackett RL, Burman JP (1946). Maximization of products by statistical method. Biometrika 33: 305-325.
Prakash K, Prajapati S, Ahmad A, Jain S, Bhakuni V (2002). Unique oligomeric intermediates of bovine liver catalase. Protein Sci. 11:46–57.
Rogalski J, Fiedurek J, Gromada A (1998). Purification of extracellular catalase from *Aspergillus niger*. Acta Microbiol. Polonica 47(1):31-43.
Scandalios JG (1968). Genetic control of multiple molecular forms of catalse in maize. Ann. New York Academy Sci. 151: 274–293.
Terzenbach DP, Blaut M (1998). Purification and characterisation of a catalase from the nonsulfur phototrophic bacterieum *Rhodobacter sphaeroides* ATH 2.4.1 and its role in the oxidativestress response. Arch. Microbiol. 169(6): 503-508.
Tsai HH, Sunderland D, Gibson GR, Hart CA, Rhodes JM (1992). A novel mucin sulphatase from human faeces: its identification, purification and characterisation. Clin. Sci. 82: 447-454.
Zaatri A (2002). Techniques de la recherche opérationnelle, ed. TOP color, Constantine, Algeria.
Zamocky M, Furtmüller PG, Obinger C (2008). Evolution of Catalases from Bacteria to Humans. Antioxid. Redox. Signal. 10(9):1527-1548.
Zhang Z, Henderson C, Gurr SJ (2004). *Blumeria graminis* secretes an extracellular catalase during infection of barley: potential role in suppression of host defence. Mol. Plant Pathol. 5 (6): 537-547.