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Full Length Research Paper

Milk-clotting fungus enzymes production: A sustainable development approach based on whey recycling

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Five species of *Aspergillus* fungus: *Aspergillus niger, Aspergillus flavus, Aspergillus awamori, Aspergillus tubingensis* and *Aspergillus tamarii*, and one *Penicillium*: *Penicillium pinophilum* were isolated from Algerian soil and identified using both microscopic observation and ICT sequence identification. Their potential milk-clotting activity was demonstrated and attributed to an acidic protease activity. For the culture and enzyme production from the six selected fungi, an approach compatible with sustainable development was developed. A whey (cheese industry secondary product) based culture medium was designed and optimised for each species using first Plackett–Burman and then Box and Wilson statistical experiments. The studied factors were pH, stirring, lactose, yeast extract, peptone, $CaCl_2$ and salts: $MgSO_4$ and $FeSO_4$. Once the selected factors were optimised, the whey based fermentation mediums were used for the production of acidic protease. All molds were shown to be able to produce acidic protease directly in their culture medium and to generate milk clotting within 15 min. The only exception was *A. awamori*, which exhibits a good acidic protease activity but no clotting capability.

Key words: Acidic protease, *Aspergillus*, experimental design, milk-clotting enzyme, optimization, *Penicillium*.

INTRODUCTION

White water from cheese industry discharged into environment should not be neglected in terms of pollution due to their richness in organic matter among others in the whey (Bouille et al., 2004), which alone accounts for 85% of milk processed into cheese. Many works on the value of whey offered new and diverse solutions for the extraction of microbial proteases from bacteria, fungi and yeast using processes like solid-state or liquid medium fermentation (Potumarthi et al., 2007).

Aspergillus and *Penicillium* are common species found in the environment, are medically and commercially important (Reetz, 2004) since some of their enzymes can be easily produced and used in different fields. For example, *Penicillium* and *Mucor* are almost exclusively used for the commercial production of extracellular acidic proteases (Thakura et al., 1990; Chrzanowska et al., 1995; Wong et al., 2008) necessary to the cheese industry milk clotting step and in these recent years other strains like *Rhizopus* (Chun-Chang et al., 2009) and

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Aspergillus (Fezouane-Naimi et al., 2010) prove interesting as a producer of rennet-like enzyme.

In this study, five species of *Aspergillus* fungus: *Aspergillus niger, Aspergillus flavus, Aspergillus awamori, Aspergillus tubingensis* and *Aspergillus tamarii*, and one species of *Penicillium*: *Penicillium pinophilum* were used. All these fungi are known to have proteolytic activity on milk agar and might be grown on whey based medium. Indeed, whey contains a large quantity of water soluble carbohydrates such as lactose and has already been used in various research works as a carbon source (Assenat and Luquet, 1985; Mechakra et al., 1999). This last point is of particular interest in Algeria and other North Africa countries since the whey is directly eliminated into streams, impacting strongly on the environment.

These fungi are excellent candidates for sustainable development, producing acidic proteases for milk clotting (cheese production) while using the cheese industry wasted whey.

Metabolite concentrations and growth of mold are strongly influenced by medium composition, such as the carbon and nitrogen source (Madigan and Martinko, 2007). The present article reports about the process optimization, using statistical experimental designs, for the six selected fungi to be grown using whey, through the optimization of biochemical and physical parameters including media formulation and culture conditions. The methodology was based on Plackett–Burman and Box and Wilson designs (Plackett and Burman, 1946).

MATERIALS AND METHODS

Reagents

All reagents used were of analytical-reagent grade and solutions prepared using Milli-Q water (Purelab option Q, ELGA). Albumin from bovine serum, calcium chloride, D-Tyrosine, ferrous sulfate, folin-Ciocalteux phenol reagent, glucose agar, lactose, magnesium sulfate, peptone, potatoes dextrose agar, potassium phosphate monobasic, sodium citrate, trichloreacetic acid (4% (V/V)), Sabouraud 2% and yeast extract were obtained from Sigma-Aldrich (France). Bio-Rad protein assay reagents were purchased from Bio-Rad Laboratories GmbH (Germany).

Analytical methods

Acidic protease assay

Enzyme activity was measured by the method of Folin-Ciocalteu. Briefly, 0.5 mL of enzyme solution (purified enzyme or culture extract) were added to 0.5 mL of phosphate buffer (0.1 M, pH 4) and 1.5 mL of casein 2.5% in sodium citrate 0.02 M, and incubated at 40°C for 60 min. The reaction was then stopped by the addition of trichloro-acetic acid and the amount of released tyrosine determined (Lenoir et al., 1979). One unit of enzyme activity corresponds to 1 µg of tyrosine released per mL and per hour.

Proteins were quantified using the Bradford method, using bovine serum albumin as standard.

Biomass determination

Biomass was collected from culture medium through paper filtration (Whatman paper no. 1). The filtrate was then washed with distilled water, and weighed after a 20 h drying step at 105°C.

Milk clotting observation

Sabouraud and sterile milk were mixed in a 4/1 volume ratio at 45°C, poured into Petri dishes and cool down to room temperature. Each mold was then deposited in the centre of a Petri dish and incubated for 72 h at room temperature.

The hydrolysis of casein results in the appearance of a fade white halo around the culture.

Microorganism

Strains of five of *Aspergillus* fungus: *A. niger, A. flavus, A. awamori, A. tubingensis* and *A. tamarii*, and one *Penicillium*: *P. pinophilum* were isolated from soil (Djebel El Ouahch, Constantine, Algeria)) and identified by CONIDIA (France) following their procedure MO-023, using a protocol developed by their laboratory (work on ITS sequence). The different strains were then seeded on Potato-Dextrose-Agar (PDA) medium at 25°C and monthly transferred. For short term storage, the strains were maintained on Sabouraud Agar at room temperature.

For long term storage, cultures were stocked as lyophilized spores. For that purpose, inocula were prepared in 250 mL flask using 50 mL PDA medium. After 6 days of incubation at 25°C, 50 mL of milliQ water were added. Spores were suspended under agitation with a magnetic stirrer, counted in a cell rest of malassez and stored at 4°C.

Whey based culture medium preparation

Goat whey was provided by the exploitation of Monique Perruset at Vaugneray (France) and derived from cheese. It was filtered on Whatman no. 1 paper and stored at -20°C.

The chemical composition of this whey was as follows: lactose, 50.21 g/L; crude protein, 2.80 g/L; crude fat, 2 g/L; dry, 58.27 g/L; total ash, 3.60 g/L; total nitrogen, 1.40 g/L; chlorides, 2.70 g/L; phosphorus, 0.251 g/L; calcium, 0.118 g/L and magnesium, 0.025 g/L (Lecoq, 1965).

Whey was diluted 1:1 with 0.1 M phosphate buffer, pH 4.0 or pH 5.0 to generate the basic culture medium. Then, according to the experimental design, different components were added to this medium: lactose, yeast extract, $CaCl_2$, $MgSO_4$, $FeSO_4$ and peptone.

All cultures were performed in 250 mL sterilized flasks (120°C for 20 min) containing 50 mL of culture medium in which 10^6 spores were added. Fermentation was carried out using a horizontal shaker (160 or 100 rpm) at 25°C for 4 days. For each experiment, enzyme activities, protein and total biomass concentrations were measured.

Data analysis

Plackett–Burman design

Statistical analysis were performed to identify which medium variables (factors) had a significant effect, either positive or negative, on enzyme, proteins and biomass production.

The following rest-degree model was used to evaluate each factor:

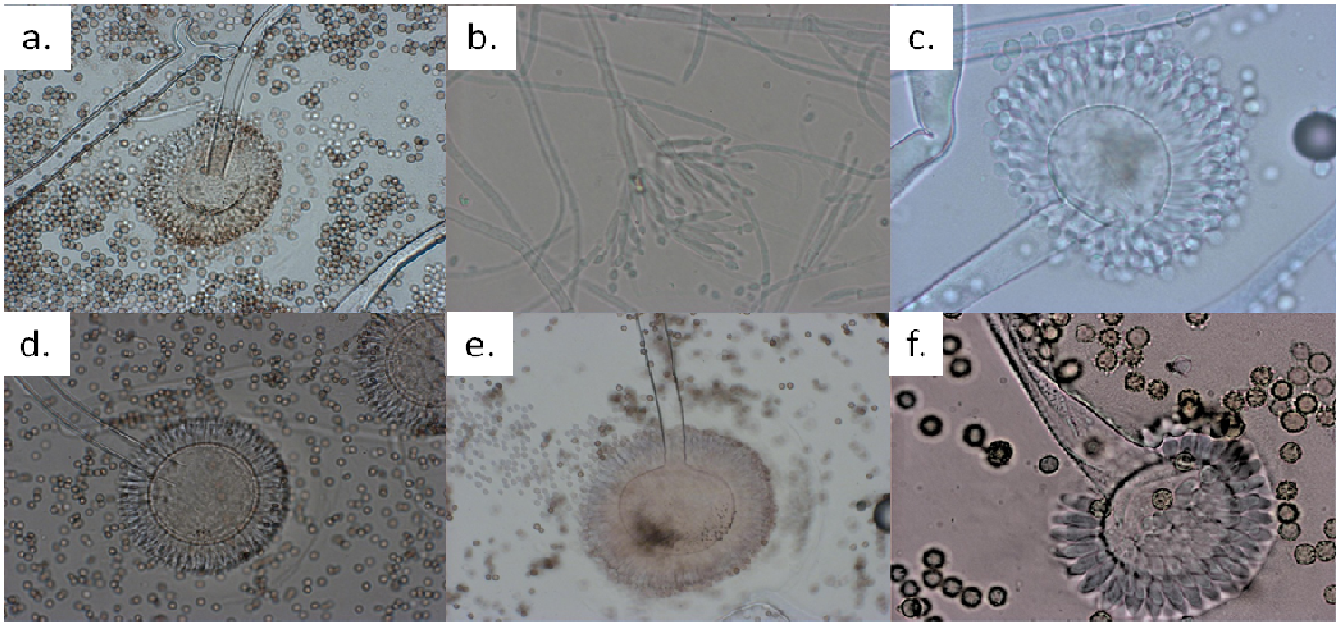

Figure 1. Optical microscopy images used for the identification of the isolated fungi: (a) *Aspergillus niger*, (b) *Penicillium pinophilum*, (c) *Aspergillus flavus*, (d) *Aspergillus awamori*, (e) *Aspergillus tubingensis* and (f) *Aspergillus tamarii*.

$Y = \text{Constant} + \sum \alpha_i X_i + \text{error}$

Wher,e α_i represents regression coefficients and X_i represents the explicative factors. The estimations of the least square of these coefficients were *p-value* observed significance levels. All coefficients having a signification probability lower than 70% were rejected, and the corresponding factors were considered without effect on the reaction.

The effect of each variable was determined as the difference between the average value of the response for the six experiments at the high level (+1) and the average value for the six experiments at the low level (-1):

$$E = \frac{\sum \textbf{response at level } (+1)}{6} - \frac{\sum \textbf{response at level } (-1)}{6}$$

Variance was defined as the average of squared errors, $V_i = \frac{\sum(\textbf{errors})2}{n}$ where, n is the number of errors.

The standard error ES was defined as the square root of the variance expressed by:

$$ES = \sqrt{Vi}$$

Calculations were performed using MINITAB 13.2 and double check using STATISTICA 2.0.

Box and Wilson design

Statistical analysis was performed to identify the selected variable optima in the case of the improvement of the production of proteolytic enzymes.

The central composite design allows studying every factor at 5 levels. It is formed by three different parts:

- 8 points of coordinates (-1, +1) which form a factorial plan
- 6 points with levels –α and +α
- 3 repetitions of the centre level 0, which is given according to the factor number of a matrix to 11 experiences (in the case of two factors) and a matrix to 17 experiences (in the case of three factors).

Such a plan allows expressing the effect of factors X1, X2 and X3 on the production of the enzyme, the proteins and the biomass in the form of an equation of the second degree:

$$Y = b_0 + b_i X_i + b_{ij} X_{ij} + b_{ij} X^2_{ij} + e$$

Where, Y is the experimental answer; b_0 is the mean value of the answers; b_i is the main effect of the factor X_i; b_{ij} is the effect of interaction of factors X_i and X_j and e is the experimental error.

The coefficients are determined by the statistical program MINITAB by-products, with regard to zero, thereby allowing the determination of coordinates coded by extremum, which transform into real values (optima) that are considered in experimental space.

RESULTS AND DISCUSSION

Identification and clotting activity

The six isolated fungi were identified by CONIDIA (France) using both microscopic observation (Figure 1) and ICT sequence identification (Supplementary Data 1). Validation of identification was made by comparing the ICT sequences with Fungi DNA international databases (NBCI). The six identified molds were *Aspergillus niger*, *Penicillium pinophilum*, *Aspergillus flavus*, *Aspergillus awamori*, *Aspergillus tubingensis* and *Aspergillus tamarii*.

The clotting ability of these six fungi was tested using a

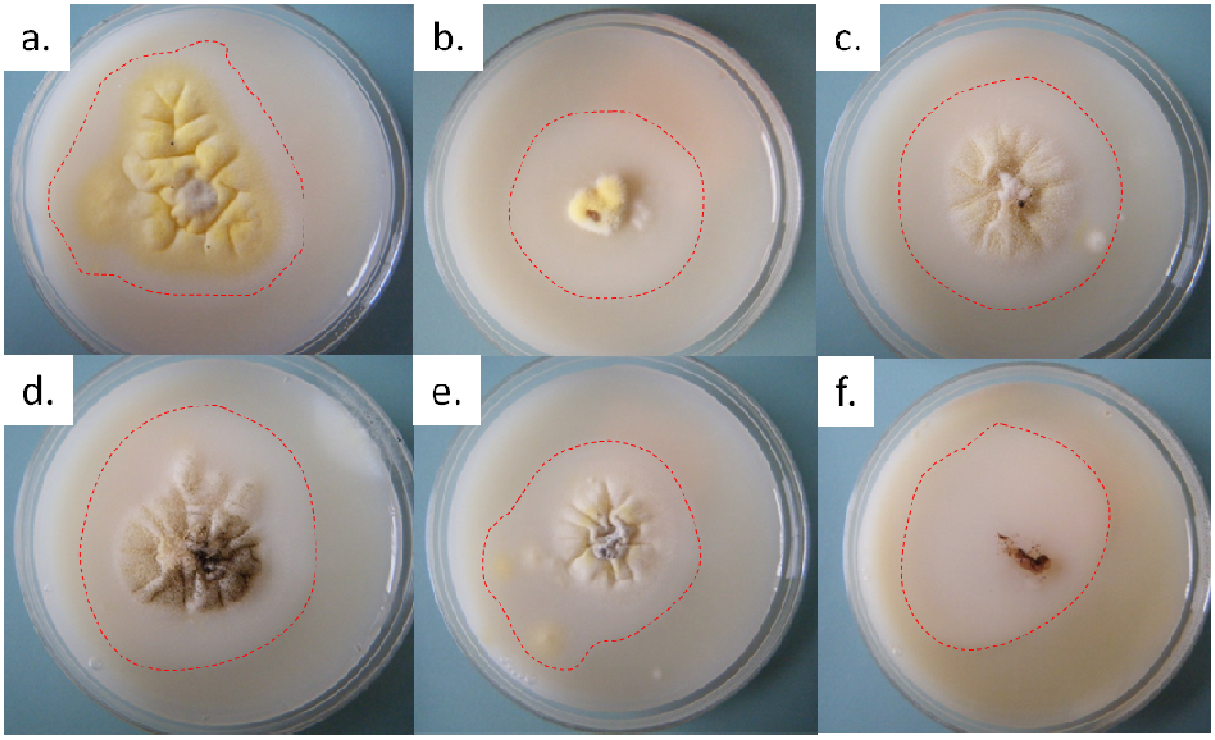

Figure 2. Milk clotting effect of the isolated fungi: (a) *Aspergillus niger*, (b) *Penicillium pinophilum*, (c) *Aspergillus flavus*, (d) *Aspergillus awamori*, (e) *Aspergillus tubingensis* and (f) *Aspergillus tamarii*. The red dotted lines are guide for the eyes to localise the clotting zone.

dedicated method based on the use of a mixed culture medium composed of nutrient agar and sterile milk. In this experiment, the clotting activity was evidenced by the presence of a fade white zone corresponding to clotted milk (see red dotted line in Figure 2). After 72 h of culture, the diameter of each halo is measured with a ruler. A halo size larger than 3 mm is considered significant.

As can be seen in Figure 2, each mold was able to generate a clotting zone surrounding the growing region with a diameter higher than 3 mm. This observation clearly demonstrates first that the isolated fungi were able to hydrolyse milk casein and secondly that the enzymes responsible for this hydrolysis were secreted by the fungi in their neighbouring medium.

These enzymes were believed to be acidic proteases. However, in the perspective of their isolation for production and characterisation, this secretion was considered as a really positive point.

Whey as a culture medium for molds' growth

Plackett–Burman design

A whey based culture medium was used in this study for the sustainable development and valorisation of resources from cheese industry. In this medium, whey is used as a basis, supplemented by different additives: lactose, yeast extract, $CaCl_2$, peptone and salts.

A Plackett and Burman design was used in order to carefully determine the effect of different parameters on the molds' culture efficiency. The composition of the culture medium varied according to the design matrix in Table 1.

The Plackett–Burman statistical design allows the study of N-1 variables with N experiences. In practice, all the experiments were carried out according to a design matrix, which was based on the number of variable to be studied (Plackett and Burman, 1946). The matrix applied to this study, describing the composition of the whey based culture medium, is shown in Table 1.

Each row represents a different experiment and each column represents a different variable. Four variables (X_3, X_6, X_9 and X_{11}) were designated as dummy variables, since their value remained constant, and were used to estimate the experimental error. The number of real variables was then reduced to N-4. Each independent variable was tested for two levels, that is, a high (+1) and a low (-1) level, which in this study means two different nutrient concentrations, two different pH values or two different agitation levels. The identification of the different factors and their high and low level values are presented in Table 2.

The effect of the selected factors on the protein and total biomass production and on the acidic protease activity was used to evaluate each factor (the raw data of

Table 1. Plackett and Burman design matrix.

Try # \ Factor	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
1	+	+	-	+	+	+	-	-	-	+	-
2	-	+	+	-	+	+	+	-	-	-	+
3	+	-	+	+	-	+	+	+	-	-	-
4	-	+	-	+	+	-	+	+	+	-	-
5	-	-	+	-	+	+	-	+	+	+	-
6	-	-	-	+	-	+	+	-	+	+	+
7	+	-	-	-	+	-	+	+	-	+	+
8	+	+	-	-	-	+	-	+	+	-	+
9	+	+	+	-	-	-	+	-	+	+	-
10	-	+	+	+	-	-	-	+	-	+	+
11	+	-	+	+	+	-	-	-	+	-	+
12	-	-	-	-	-	-	-	-	-	-	-

Table 2. The factors and their -1 and +1 level value.

Factor \ Level		Level (-1)	Level (+1)
X_1: pH		4	5
X_2: Stirring		100 rpm	160 rpm
X_3: Error		/	/
X_4: Lactose		25 g/L	50 g/L
X_5: Yeast extract		5 g/L	10 g/L
X_6: Error		/	/
X_7: $CaCl_2$		0 g/L	1 g/L
X_8: Peptone		5 g/L	10 g/L
X_9: Error		/	/
X_{10}: Salts:	Mg SO_4	0 g/L	0.2 g/L
	$FeSO_4$	0 g/L	0.1 g/L
X_{11}: Error		/	/

each experiment are presented in Supplementary Data 2).

Stirring and pH

First, the increase of the culture medium stirring from 100 to 160 rpm was shown to have a positive effect on the protein production from five molds. Thus, significant *p-values* (observed significance levels) of 84, 91, 86, 95 and 98% were obtained for *A. niger, A. flavus, A. awamori, A. tubingensis and P. pinophilum*, respectively, whereas non-significant effect on the *A. tamarii* protein production was observed.

At the same time, biomass production *p-values* of 98, 98, 99 and 99% were achieved for *P. pinophilum*, *A. flavus, A. awamori* and *A. tubingensis,* respectively, evidencing a positive effect of the stirring. Inversely, a non-significant effect of the stirring on the biomass production from *A. niger* and *A. tamarii* was observed.

The positive effect of the stirring efficiency on the protein and biomass production can be related to the improvement of the medium oxygenation, known as an important factor for the growth of aerobic micro-organisms (Botton and Breton, 1990; Martinelli and Kinghorn, 1997).

Using this first row of experiment, a 160 rpm stirring was selected and used for all subsequent cultures. Increasing the culture medium pH from 4.0 to 5.0 also had a positive effect on the protein production by *A. niger* and *A. tamarii* with *p-values* of 82 and 83%, respectively. This factor also positively influenced the biomass production from *P. pinophilum* to *A. tubingensis* with *p-value*s of 77 and 80%, respectively. For all other molds, the effect of the pH variation was found to be non-significant with *p-value*s below 70%. Then, since the effect of the increasing pH value was non-significant for a majority of molds, the pH of all fermentation solution was maintained at 4.

Yeast extract

The addition of yeast extract, which is a good stimulus for protein synthesis (Larpent and Larpent-Gourgaud, 1985), to the culture medium demonstrated positive effect on the protein production of three molds with *p-value*s of 96, 87 and 99% for *A. niger*, *P. pinophilum* and *A. flavus,* respectively. For all other molds, a non-significant effect of the yeast extract on the protein production was obtained. According to biomass production, the effect of yeast extract was mitigated. A positive effect was observed for *A. awamori* (*p-value* of 99%); while a negative effect was achieved for *A. flavus* (*p-value* of 93%) and *A. tubingensis* (*p-value* of 83%).

About the enzyme activity, the addition of yeast extract has a positive effect only on *P. pinophilum* with *p-value*

Table 3. Fungi specific whey based culture medium components.

Specie	Selected medium composition
A. niger	Whey + yeast extract + peptone + salts
A. flavus	Whey + yeast extract + peptone
A. awamori	Whey + yeast extract + peptone
A. tubingensis	Whey + yeast extract + lactose + salts
A. tamarii	Whey + peptone + $CaCl_2$
P. pinophilum	Whey + yeast extract + lactose + $CaCl_2$

of 70%. In all other cases, yeast extract has a non-significant effect.

Peptone

Peptone can be used as an essential source of nitrogen (amino acids and ammonium ions) (Scriban, 1993) for molds' growth. In this study, peptone addition showed a very significant positive effect on the protein production by *A. flavus*, *A. awamori* and *A. tamarii* with *p-value*s of 93, 70 and 80%, respectively. It also has a positive effect on the biomass production of molds, by *P. pinophilum* and *A. flavus*, with *p-value*s of 76 and 92%, respectively. In all other cases, peptone has a non-significant effect except for the protein production by *P. pinophilum* (where it has a negative effect with a *p-value* of 75%).

The acidic protease activity from *A. niger* and *A. tamarii* was also influenced by the addition of peptone with *p-value*s of 99 and 87%, respectively.

Lactose

In addition to whey, lactose can be added to the culture medium as a supplementary source of carbon. Here, the addition has a positive effect only on the biomass production, by *A. flavus*, with a *p-value* of 97%, and on the protein production of *A. tubingensis* with a *p-value* of 79%. Lactose also has negative effects on protein production by *A. flavus, A. tamarii* and *P. pinophilum*, and on biomass production by *A. tubingensis* and *P. pinophilum*.

The addition of lactose as a carbon source also has a positive effect on the enzyme production by *P. pinophilum* with a *p-value* of 76%. In all other cases, lactose has a non-significant effect.

Salts

The presence of different salts (magnesium sulphate and iron sulphate) has very low effect on the protein synthesis and biomass production of all molds and a positive effect on the enzymatic activity of only *A. niger* (with a *p-value* of 84%).

These results can be explained by the fact that yeast extract and peptone already provides sufficient salts concentrations for the molds to grow and produce proteins.

$CaCl_2$

The use of $CaCl_2$ has a positive impact on biomass production by *A. niger* and *A. flavus* with *p-value*s of 72 and 83%, respectively, but has a positive effect only on protein production by *A. niger* (*p-value* of 86%) and no effect on the enzyme activity.

As the final outcomes of this first statistical study, the equations of the biomass production, protein production and acidic protease activity were calculated according to the method described in the study's data analysis.

For biomass production, the equations were $Y= 19.117 + 0.650 X_4 + 1.383 X_7 + 0.517 X_8 + 0.850 X_{10}$ for *A. niger*; $Y= 15.167 + 4.400 X_4 + 2.933 X_8$ for *A. flavus*; $Y= 20.733 + 0.100 X_4 + 1.400 X_5 + 0.033 X_8$ for *A. awamori*; $Y= 15.367+ 0.133 X_7 + 1.067 X_{10}$ for *A. tubingensis*; $Y= 17.533 + 1.500 X_4 + 1.967 X_7 + 2.400 X_8$ for *A. tamarii* and $Y= 8.817 + 0.317 X_5 + 0.683 X_7 + 0.850 X_8 + 0.150 X_{10}$ for *P. pinophilum*.

For protein production, the equations were $Y= 74.21 + 12.92 X_2 + 22.20 X_5 + 13.42 X_7$ for *A. niger*; $Y= 178.90 + 13.85 X_2 + 45.56 X_5 + 15.47 X_8$ for *A. flavus*; $Y= 59.54 + 8.97 X_2 + 4.23 X_5 + 5.79 X_8$ for *A. awamori*; $Y= 99.32 + 45.36 X_2 + 24.48 X_4 + 11.30 X_5 + 13.04 X_{10}$ for *A. tubingensis*; $Y= 133.93 + 1.74 X_5 + 7.36 X_8$ for *A. tamarii* and $Y= 181.66 + 104.73 X_2 + 55.62 X_5$ for *P. pinophilum*.

For enzyme activity, the equations were $Y= 670.17 + 196.49 X_2 + 108.19 X_8 + 32.75 X_{10}$ for *A. niger*; $Y= 573.84 + 127.61 X_2 + 14.30 X_8$ for *A. flavus*; $Y= 681.10 + 137.24 X_2$ for *A. awamori*; $Y= 627.99 + 189.40 X_2 + 0.57 X_8 + 2.10 X_{10}$ for *A. tubingensis*; $Y= 609.10 + 118.67 X_2 + 9.20 X_7 + 112.03 X_8$ for *A. tamarii* and $Y= 668.83 + 160.06 X_2 + 68.86 X_4 + 59.88 X_5 + 32.02 X_7$ for *P. pinophilum*.

From this statistical study, molds' specific compositions of the culture medium were proposed (Table 3). As such, the concentrations of the different factors (except whey) will be optimised in the following study and used for fermentation.

Box and Wilson design

After the factors' selection, using the Plackett–Burman design (Table 3), and for a more precise location of the different optima, the factors were evaluated again using a central composite design.

Indeed, in order to determine a quadratic polynomial, each factor k must be studied, at least, at 3 levels, requiring a 3^k plan with an impressive number of combinations. Here, the central composite Box and Wilson design (Box and Wilson, 1951) offers an economic and

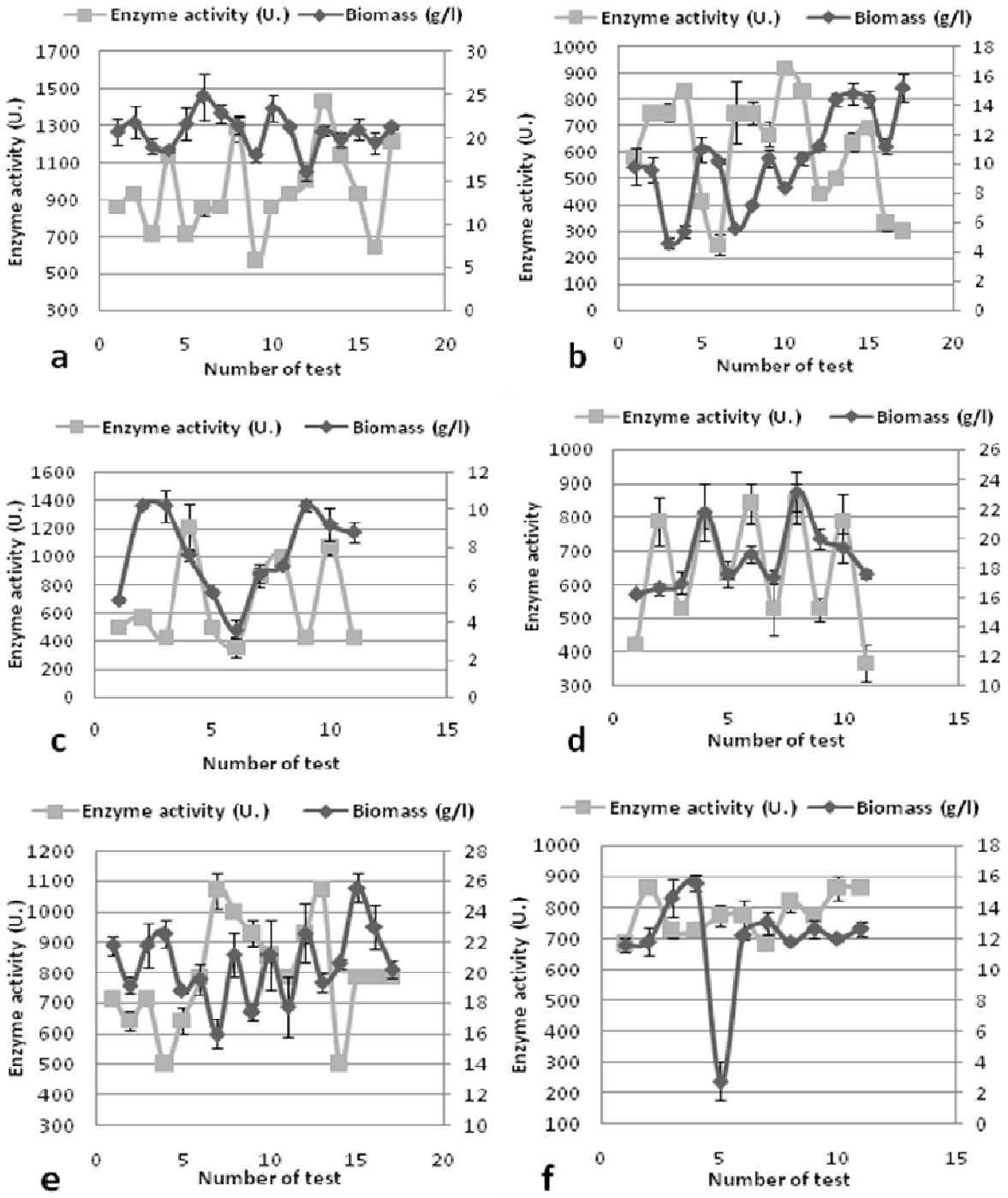

Figure 3. Variation of biomass and enzyme activity according to the design of Box and Wilson of the isolated fungi:(a) *Aspergillus niger*, (b) *Penicillium pinophilum*, (c) *Aspergillus flavus*, (d) *Aspergillus awamori*, e) *Aspergillus tubingensis* and f) *Aspergillus tamarii*.

effective alternative.

The concentrations of the selected factors used for the Box and Wilson experiments are presented in *Supplementary Data 3* and the variation of biomass and enzyme activity of six strains is shown in Figure 3.

The optimums were determined by the statistical program MINITAB through the achievement of a quadratic model for each parameter and each strain. Then, the reaction outcomes (biomass, proteins and activity) can be approximated by a polynomial equation of the second

Table 4. Optimum culture durations, fermentation outcomes and milk-clotting test results.

Specie	Culture time (h)	Protein concentration (µg/ml)	Biomass (g/l)	Enzymatic activity (U)	Clotting time (min)
A. niger	96	232.48	21.20	1357.14	8 (flake)
A. flavus	144	209.82	17.80	1533.33	8 (clot)
A. awamori	120	243.08	19.20	1285.71	-
A. tubingensis	72	248.81	6.80	2422.22	10 (flake)
A. tamarii	144	199.75	18.80	2577.77	5 (clot)
P. pinophilum	80	371.72	9.80	1428.57	15 (flake)

degree:

$$\mathbf{Y = b_0 + b_j X_i + b_{IJ}\, X_{IJ} + b_{IJ}\, X_{IJ}^2 + e}$$

Where, Y is the measured response (either biomass, protein or activity); a_i is the linear effect of each co-efficient of the factor and e is a random error.

Using the results, the reduced polynomial equations were written and the answers to the equations were represented in three or two dimensions depending on the strain studied. From these graphical representations, the extremums of these functions were determined, and the stationary points of all strains were calculated. These stationary points allowed the calculation of the optimized concentration of the different factors (see *Supplementary Data 4* for the detailed values). As can be seen from the *Supplementary Data 3* and 4, the determined optimum concentrations were all included in the studied concentration ranges, giving a strong weight to the Box and Wilson study.

Fermentation results

The optimized medium compositions were finally used to produce acidic protease from the six different molds. The kinetic of the three measured parameters (biomass, proteins and activity) was followed at least for six days and the optimum fermentation time was selected for the concomitant maximum production of protein and the maximum acidic protease activity in the medium. Table 4 reports these optimum culture durations and the fermentation outcomes. As can be seen, the strains have really different culture optimum duration, from 3 to 6 days, but are able to generate consistent levels of protein and acidic protease activity.

As a final result of our whey based fermentation study, the milk-clotting activity of the culture medium was tested. For that purpose, filtrated culture mediums were mixed with whole milk (without any further additives) and the clotting time was measured (Berridge, 1945). The results of this clotting study are presented in Table 4. As can be seen, all molds were able to generate clot within 15 min except *A. awamori*. This is a really promising result for the use of these fermentation products in local cheese industry.

Conclusion

Six fungi molds were extracted from Algeria soil, isolated and identified, and were shown to exhibit a potential milk-clotting activity.

Interestingly, in order to contribute to sustainable development and valorisation of resources from the cheese industry, whey was valuably exploited as a carbon source for the growth of the six fungi species.

A Plackett and Burman design was used to select the additives necessary for the growing of each mold, using this whey based culture medium, and paying attention to the biomass and protein production of the milk-clotting acidic protease activity.

On the basis of this Plackett and Burman study, the selected microorganisms appeared to preferentially require the addition of nitrogenous organic molecules (Botton and Breton, 1990) from yeast extract and peptone, and seems to be able to grow using whey or whey plus lactose as a carbon source (Regnault, 1990).

Following this first statistical study, the selected factors for each strain were included in a Box and Wilson central composite design and used to optimize the medium for the kinetic study of the biomass and the synthesis of milk-clotting enzymes by the selected fungi. Once the fermentation conditions were optimised, the different molds were successfully used to produce acidic protease activity, and 5 over 6 were found to directly generate culture medium with milk-clotting activity.

The next step in this sustainable development approach will be the purification and characterisation of the produced milk-clotting acidic protease.

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REFERENCES

Assenat L, Luquet FM (1985). Vache Brebis Chèvres Les Laits de la mamelle à la laiterie. Lavoisier, Paris, France.

Berridge NJ (1945). The purification and crystallisation of rennin. Biochem. J. 39(2): 179-186.

Botton B, Breton A (1990). Moisissures utiles et nuisibles, importance industrielle. Masson, Paris, France.

Bouille E, Dubois V, Egal M, Herpin P, Porterie P, Senesse O, Vales R (2004). Traitement, épuration et valorisation des effluents d'une fromagerie. From http://hmf.enseeiht.fr/travaux/CD0405/beiere/4/html/index.htm.

Box GEP, Wilson KB (1951). On the Experimental Attainment of Optimum Conditions. Statistical Methodology. J. Roy. Statistical Soc. 'b'. 13(1): 1-45.

Chun-Chang C, Yen-Ching C, Chien-Chen L, Wen-Hwei H (2009). Purification and characterization of new Rhizopuspepsin from *Rhizopus oryzae* NBRC 4749. J. Agric. Food. Chem. 57: 6742-6747.

Chrzanowska J, Kolaczkowska M, Dryjanski M, Stachowiak D, Ponanowski A (1995). Aspartic proteinase from *Penicillium camemberti*. Purification Proprieties and substrate specificity. Enzyme Microb. Tech.17: 719-724.

Fezouane-Naimi F, Mechakra A, Abdellaoui R, Nouani A, Magagi Daga S, Marou Alzouma A, Gais S, Pennincks MJ (2010). Characterization and cheese-making properties of Rennet-Like enzyme produced by a local Algerian isolate of *Aspergillus niger*. Food Biotechnol. 24: 1-12

Larpent JP, Larpent-Gourgaud M (1985). Eléments de Microbiolgie. Hermann, Paris, France.

Lecoq R (1965). Manuel d'analyse alimentaire et d'Expertises Usuelles. Lavoisier, Paris, France.

Lenoir J, Auberger B, Gripon JC (1979). Les caractères du système protéolytique de *Penicillium caseiculum*. III. Caractérisation d'une protéase acide. Lait. 59 : 244-268.

Madigan M, Martinko J (2007). Biologie des microorganismes Brock. Pearson, Paris, France.

Martinelli SD, Kinghorn JR (1997). *Aspergillus* 50 Years on Progress in Industrial Microbiology. Elsevier, London, UK.

Mechakra A, Auberger B, Remeuf F, Lenoir J (1999). Optimization of a culture medium for acid proteolytique enzyme production by Penicillium camemberti. Sciences des aliments, 19(6): 663-675.

NBCI. National Center for Biotechnology Information. From http://www.,cbi.nlm.nih.gov/

Plackett KL, Burman JP (1946). The design of optimum multifactoriel experiments. Biometrika, 33: 305-325.

Potumarthi R, Subhakar CH, Jetty A (2007). Alkaline protease produced by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. Biochem. Eng. J. 34: 185-192.

Reetz MT (2004). Methods in enzymology. Elsevier, Academic press, San Diego, California. USA.

Regnault JP (1990). Micrbiologie générale. Décarie Vigot, Paris, Fance.

Scriban R (1993). Biotechnologie. Lavoisier, Paris, France.

Thakur MS, Kranth NG, Krishna N (1990). Production of fungul rennet by Mucor miehei using solid stat fermentation. Appl. Microbiol. Biotechnol. 32: 409-413.

Wong Y, Xue W, Sims AH, Zhao C, Wang A, Tang G, Qin J, Wang H (2008). Isolation of four pepsin-like protease genes from Aspergillus niger and analysis of the effect of disruptions on *heterologous laccase* expression. Fungal. Genet. Biol. 45:17-27.