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Response surface methodology optimization of milk-clotting protease produced by *Pleurotus sajor-caju* strain CTM 10057 and its technico-economical evaluation

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ABSTRACT/RESUME

Abstract:*The article purpose is to scale-up the bioreactor design for the serine protease from Pleurotus sajor-caju strain CTM 10057 (called SPPS) production to be profitably used in cheese-making production. The SPPS enzyme has been optimized using central composite design (CCD) statistical design analysis. The optimal conditions are: a carbon source of 15 g/L, a nitrogen source of 2 g/L, agitation of 160 rpm at pH = 5.6. This production is scaled-up using submerged fermentation. Under the optimized conditions, protease yield 75,000 U/mL was 6.5 folds higher than those obtained by the use of the initial conditions (11,600 U/mL). The separation was carried out throughout centrifugation followed by a concentration with evaporation, after which this enzyme was tested in the enzymatic milk coagulation process. According to its milk-clotting ability, it could be used in the cheese industry, as well as other food industries. Interestingly, the economic analysis of this planned bioprocess is leased at a positive gain. The estimated SPPS price will be competitive in comparison with commercial enzymes (Mucorpepsine and Presura). So, SPPS enzyme can be used in cheese making and its process is profitable.*

I. Introduction

The chemical process industry (CPI) is involved in manufacturing widespread variety products that improve our lives quality and generate the businesses revenue. Generally, chemical engineers encounter a variety of chemical process flow diagrams [1]. According to globalization, the industries militate for the chemical engineering evolution toward the green processes. These will create new challenges. It can also put complex issues at the global level at the molecular, product and process levels. For that, chemical engineers will play a crucial role in developing new processes that require new design methods for the implementation of effective new technology. This new design is based on an integrated biocatalyst and process engineering approach. In this way, a directed development can be realized and the chances of implementing a commercially successful process can be greatly improved [2]. This multi-

scale approach is used in biotechnology and bioprocess engineering to well understand and control biological tools such as enzymes and microorganisms. It can also use to produce structured products [3]. To protect our environment, the exploitation of renewable and green materials is necessary for future chemical processes. These factors are necessary motorists in industrial biotechnology implementation. Indeed, the bioprocessing is an innovative area that aims the creation of an entirely range of products. Despite these scientific advances, biocatalysts are still often limited due to a poor transition from the laboratory to the industrial scale.

Today, this discipline has met many challenges such as environmental protection and safety [4] to meet the current and future needs of the chemical industries. These sciences are based on better understanding and control of biological tools such as enzymes and microorganisms and to produce structured products. This approach covers nanoscale (molecular and genomic processes, and metabolic transformation), micro-scale (respectively, enzymes in integrated enzyme systems, biocatalyst environment, and active aggregates), the average scale for unit operations (bioreactors, fermenters, exchangers, separators, etc.), and macroscales and megascals (respectively, for units and plants, and for interaction with the biosphere). In addition, the ability to think across multiple scales encourages chemical engineers particularly to elucidate the understanding of molecular and cellular biology and its larger-scale manifestation using the principles of chemical engineering [5]. This science is based on the use of biological systems (bacteria or fungi) for the manufacture, transformation or degradation of molecules through enzymatic processes or fermentation for industrial purposes. Subsequently, enzymes are proteins that greatly accelerate the biochemical reaction in the cell. Indeed, the specificity and the ability to work under reasonably mild conditions make enzymes the preferred catalysts in various applications. The multi-scale approach therefore has tremendous potential for linking marketing, modeling and optimization tools to create the best chemical for each customer or product. Thus, in addition to the well-known notions of unit operations, coupled to heat transfers, mass and momentum, the traditional tools of chemical engineering, as well as the fundamental principles of chemical engineering and process (separation, catalysis, thermodynamics, process control, economics, considerations, etc.) are beneficial for the development and success of this engineering. This involves the synthesis of nano- and micro-structures, design, scaling or reduction, control and optimization of industrial processes through physico-biochemical separations and by chemical, catalytic, biochemical, electrochemical, photochemical and agrochemical reactions [3].

Food industry has an important existence in our lives especially cheese product industry. Coagulating enzymes are an absolute necessity for the production of cheese varieties. Industrial enzymes can be derived from a wide variety of plant, animal or microbial sources. Rennin, the milk-clotting enzyme used in cheese-making is obtained from the stomach contents (rennet) of the unweaned calf. Because of the difficulty in acquiring sufficient quantities of rennet for the cheese industry, other sources for the enzyme have been sought for many years. Various animals, plants and microbial proteases have been suggested as milk coagulants [6]. Thanks to their facility of separation by centrifugation, the fungi proteases are chosen for this application. Studying the scale-up of SPPS at an industrial scale from laboratory data. Thus, laboratory-scale data can be safely used to develop economic evaluations of submerged fermentation process, as the tendency of the process is to maintain extraction yield at an increasing scale. Raw materials such as fungi nutrients represent 60 to 80% of the protease price cost produced by fermentation [7]. So, they are chosen according to the following criteria: (i) accounting with the regulations in force in modern countries regarding the standards of products intended for food: absence of antiseptics, pesticides, heavy metals, etc (ii) stable supply in quantity and quality throughout the year, and (iii) costs as low as possible, including transportation.

Virtually all regularly used products come from the agricultural and food industries [8]. The standard size for a larger scale process is from 1 L up to 70 L than for a pilot scale. Ideally the fermentation broth at the end of production should be a clear solution of protease with a precipitate formed of producing microorganisms. In practice, the broth will often contain 1% dry cell material, 0.1 to 0.5% enzymes, 1 to 2% nutrient residues and fermentation medium compounds. Enzymatic protein content based on protein addition can reach 10% [9].Therefore, the main objective of this study is to scale-up the SPPS production to be used in cheese-making industry and to estimate its industrial production cost.

II. Materials and Methods

II.1. Experimental Design and Statistical Analysis

SPPS optimized activity was educated using the central composite design (CCD). The major factors are carbon concentration (X_1), nitrogen concentration (X_2), agitation (X_3) and pH (X_4). They were screened as variables at five levels (-2, -1, 0, 1, and 2) with a total number of 37 experiments. The latter included 23 full factorial design experiments (runs 1–8), six axial points

(runs 13–18), and twelve replicates in the domain center (runs 25-36) to estimate the variability of the experimental data. The data were analyzed using the statistical analysis Statistical Package for Social Sciences (SPSS, Version 11.0.1, 2001, LEAD Technologies, Inc., USA), and the response surface was generated using the Microsoft Excel (Version 2003, Microsoft office, Inc., USA) program. The regression model was constructed based on the SPSS procedure, which initially considers all the factors involved and then applies a step-by-step elimination of the non-significant ones. The response values of protease activity (U/mL). The interaction of variable was adjusted by the least-square method using the quadratic model. This model can be represented by the following polynomial equation: where X_1, X_2, X_3 and X_4 refer to the coded factors studied (Table 1), b_0 to the intercept, b_1, b_2, and b_3 to linear coefficients, b_{11}, b_{22}, and b_{33} to squared coefficients, and b_{12}, b_{13}, and b_{23} to interaction coefficients.
The model coefficients were estimated using multi linear regression. The significance of the coefficients was evaluated by a multiple regression analysis based on the F-test with unequal variance ($P < 0.05$). To check the compatibility of the proposed model with the experimental data obtained, an analysis of variance was performed.

II.2. Analytical Methods

In order to correct non-enzymatic effects, separate blanks for each sample were established $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{21} + b_{22}X_{22} + b_{33}X_{23} + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$.

II.3. Preparation of Inoculums

The *Pleurotus sajor-caju* strain CTM 10057 was cultured on Potato Dextrose Agar (PDA) composed (in g/L) Potato infusion, 200; Dextrose; 20, and Agar, 20 at 30 °C, pH 5.6 for 36-h. The colonies appeared on the plate were picked up are inoculated into 100 mL optimized medium and incubated at 30 °C, for 24 h with shaking at 160 rpm in 1000-mL Erlenmyer flask. The culture broth was then used as inoculums for the submerged fermentation.

II.4. Submerged Fermentation

Samples were prepared as described above, which were then combined with 50 mL of L-medium composed of Lentil flower 15 g/L, Yeast extract 2 g/L, Glucose 10 g/L, KH_2PO_4 1 g/L, and K_2HPO_4 1 g/L in a 500-mL flask; the whole mixture was steamed and sterilized in autoclave at 121 °C for 15 min. This contained medium was inoculated with 5 mL (5%, v/v) of fungi inoculums prepared above and incubated at 30 °C at pH 5.6 with shaking at 160 rpm for 24 h. At the end of incubation, the culture medium was filtered through the cotton cloth, and then it was centrifuged at 4 °C for 5600×g and 20 min and filtering through a syringe filter (0.45 µm) to remove the fungi cells. The liquid broth of the crude enzyme was used to assay for milk-clotting activity. A lab-scale batch fermentation unit was used for the extraction of SPPS from *Pleurotus sajor-caju* strain CTM 10057. The schematic diagram of the system setup is shown in Figure 3. In this study, 5 g of Lentil flower was used. Two operating conditions were investigated.

II.5. Milk-Clotting Activity of SPPS.

Here, the SPPS production was finished at large scale. Formerly, it was clarified by centrifugation (5600×g, 20 min) to have enzyme biomass free. The ability of milk-clotting of SPPS was tested at 37 °C using the protocol described by Arima et al. (1970) [10]. Different concentrations of SPPS proteases were added in 5 mL "Délice" skimmed milk and incubated in an oven at 37 °C for 24 h for their coagulating activities. Observations of the coagulation process were made during the incubation time.

II.6. Comparative Study Physical Properties of SPPS and Mucorpepsin Coagula

II.6.1. Viscosity

The relative viscosity is measured at 4 °C using an Ostwald viscometer.

$$Rv = \frac{t}{te}$$

Where *Rv*, *t*, and *te*, refers to relative viscosity, flow time of the milk drink, and time of flow of water, respectively [11].

II.6.2. Color

The color and clarity were evaluated by a colorimeter (Konica Minolta Chroma Meter CR-5, Tokyo, Japan) to determine the CIELab coordinates (L*, a*, and b*). The instrument was calibrated (L* = 93.87, a* = 0.18, and b* = 2.71) and the L*, a*, and b* values indicated respectively whiteness, redness, and yellowness. For more appropriate color determination, the index of whiteness (WI) was also calculated according to the following formula [12]:

$WI = L^* - 3b^*$

II.6.3. pH

The pH of the coagulum was measured using a previously calibrated pH meter (744 Metrohm pH meter) at 20 °C [13].

II.7. Statistical Analysis

The analyses were done in triplicate and the data obtained were subjected to student's t-test to compare physical quality of SPPS and Mucorpepsin coagula. Differences were considered to be significant at $P < 0.05$. Statistical analysis was done with SPSS software (Statistical Package for Social Science, version 23.0).

II.8. Cost Estimation

The cost effectiveness of protease production was estimated. The determination of different steps was done. First, the production step using submerged fermentation. Second, the biomass separation using centrifugation. Third, the enzyme concentration by evaporation. The evaluation of SPPS cost production was done using the Turton methodology presented by Rosa and Meireles [14]. Based on factors influence in enzyme production, it was divided into three categories: direct costs, fixed costs and general expenses. Direct costs are expenses that depend on the production rate, such as materials. Fixed costs are constant even during process interruption and include equipment taxes costs. General expenses are related to business maintenance such as research, administrative cost.

III. Results and Discussion

III.1. Optimization of Protease Production using the Central Composite Design

The experimental design methodology was used to determine the optimal values of the selected variables and enhance the protease production yield. The central composite design was applied, and the experimental responses are presented in Table 1.

Table 1: *The CCD plan in actual values along with experimental response for protease production optimization.*

Experimental number	X1	X2	X3	X4	Protease activity (U/mL)
1	5	2	150	5.8	5,410
2	5	2	150	6.2	4,571
3	5	2	200	5.8	5,471
4	5	2	200	6.2	4,579
5	5	4	150	5.8	4,759
6	5	4	150	6.2	5,741
7	5	4	200	5.8	6,471
8	5	4	200	6.2	7,541
9	15	2	150	5.8	10,000
10	15	2	150	6.2	9,700
11	15	2	200	5.8	9,200
12	15	2	200	6.2	9,120
13	15	4	150	5.8	9,100
14	15	4	150	6.2	8,645
15	15	4	200	5.8	7,500
16	15	4	200	6.2	6,700
17	0	3	180	6.0	842
18	20	3	180	6.0	7,510
19	10	5	180	6.0	5,240
20	10	1	180	6.0	4,600
21	10	3	120	6.0	4,732
22	10	3	250	6.0	4,755
23	10	3	180	5.6	10,600
24	10	3	180	6.4	5,700
25	10	3	180	6.0	8,400
26	10	3	180	6.0	7,200
27	10	3	180	6.0	8,300
28	10	3	180	6.0	8,100
29	10	3	180	6.0	8,500
30	10	3	180	6.0	8,700
31	10	3	180	6.0	8,150
32	15	2	160	5.6	8,200
33	10	3	180	6.0	8,400
34	10	3	180	6.0	8,340
35	10	3	180	6.0	8,200
36	10	3	180	6.0	8,320
37	10	3	180	6.0	8,430

The F-test (ANOVA) was used to identify the statistical significance of the second-order model (Table 2).

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Table 2. *ANOVA analysis for protease production in central composite design experiments.*

Model	Sum of Squares	Degree of freedom	Mean Square	F value	Significance
Regression	1.166E+08	14	8.329E+06	5.676	
Residual	2.348E+07	16	1.467E+06		
Total	1.401E+08	30			

The regression model for protease production was highly significant ($P < 0.01$), with a satisfactory value of determination coefficient ($R^2 = 0.912$), indicating that 91.2% of the variability in the response could be explained by a second order polynomial equation:

$$Y = 155{,}273.73 + 2819{,}15*x_1 - 1{,}195.29*x_2 + 356{,}24*x_3 - 61{,}790.52*x_4 - 31.63*x_1*x_1 - 131.95*x_1*x_2 - 4.15*x_1*x_3 - 122.25*x_1*x_4 - 604.75x_2*x_2 + 3.84*x_2*x_3 + 908.75*x_2*x_4 - 0.65*x_3*x_3 - 16.15*x_3*x_4 + 5{,}068.68*x_4*x_4$$

Where Y refers to activity (U/mL), X_1 to the carbon concentration (g/L), X_2 to the nitrogen concentration, X_3 to the agitation, and X_4 to the pH.

The analyses of the quadratic model showed that the variables with the largest effects were the linear. The interaction between carbon concentration and pH, nitrogen concentration and pH could be better visualized by plotting the 3D response surface graphs. Accordingly, the response surfaces were obtained by varying the two factors while the third factor was maintained constant at its intermediate level (Figs. 1A–B). The elliptical contour plots of response surfaces showed that the interactions between the related variables are significant. Under the optimized culture conditions, the quadratic model showed that the maximum protease production yield of 11,600 U/mL could be attained when the strain was grown using carbon concentration of 15 g/L, nitrogen concentration of 3 g/L; agitation for 160 and pH 5.6. The culture medium optimized by experimental design using central composite designis composed of (in g/L)
Lentil flower 15, Yeast extract 2, KH_2PO_4, 0.5, and K_2HPO_4, 0.5 with an agitation of 160 rpm and pH = 5.6. The maximum allowed activity of 11,600 U/mL.
The culture composition is usually optimized to maintain the interaction of the various compounds. This interaction can be studied by experimental design. Two interactions are discovered: the first is the interaction between carbon source and pH (Fig. 1A) and the interaction between nitrogen source and pH (Fig. 1B).

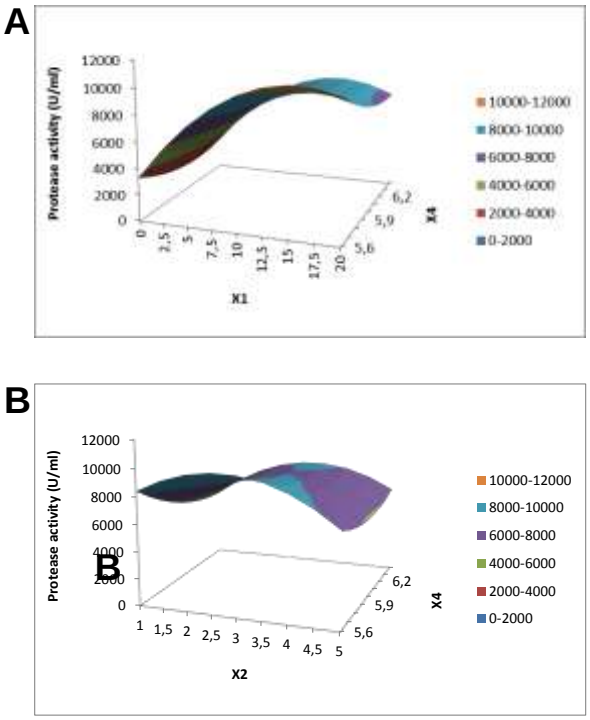

Figure 1. *Response surface plots of SPPS activity showing the interactions among the carbon concentration and pH (A), the nitrogen concentration and pH (B) by the central composite design.*

III.2. Validation of Optimized Protease Production

This validation was performed at least in triplicate in Erlenmeyer test at optimized conditions. The production yield was 16 times better than the one obtained in Sabouraud medium.

III.3. Scale-up of SPPS Production

The scale-up of this enzyme production to submerged fermentation showed a wide variation of protease activity production to 75,000 U/mL, using low coast substrate. The scale-up of this production using powdered milk, was enhanced six folds. The scale-up production can increase the size without any significant effect on the results produced. The simulations carried out consider that the production of 75,000 U/mL after 72-h of fermentation. It is also expected that the biomass produced during the fermentation would be separated from the culture medium by centrifugation. This practice should

make it possible to obtain a biomass concentrate. The protein extraction and recovery chain is based on the method described by Kalk [15]. The fermentation time is also very important since the culture medium used for a long fermentation with a high production of enzymes is different from that required for a short fermentation. Continuous and semi-continuous processes for the production of proteases are not common commercial practices, although some authors have demonstrated that it is possible to maintain high protease production over long periods of time [16]. In this study, it's better to choose the batch reactor. This process has however, simplified to eliminate the lysis steps cell and nucleic acid elimination since the studied processes correspond to an extracellular proteins production.

III.4. Performance of SPPS in Milk-Clotting

The results of the action of different concentrations of SPPS proteases and Mucorpepsin on "Délice" skimmed milk (40 mL) at 37 ° C (Fig. 2). The best concentration of SPPS was 100 µL in 5 mL commercial milk. The SPPS coagulation action was achieved after 10-h.

II.5. Comparative Study Physical Properties of SPPS and Mucorpepsin Coagula

The physical proprieties were examined using the methods announced in experimental section. The color, pH and viscosity were resumed in Table 3 and the texture was shown in Table 3, SPPS shows better properties than the commercial enzyme Mucorpepsin. Hence SPPS is a good potential candidate for milk coagulation. The comparison of physical proprieties shows that SPPS products is more performed then Mucorpepsin (commercial enzymes). The pH values found in this study for coagulum samples were in the same range to those reported by Ramasubramanian et al., 2013 [17] and Aswal et al., 2013 [18] for fermented milk and yaourt coagulum, respectively. The acidic pH of both coagulum samples reflects the activity of enzymes during the milk-clotting reaction.

With color data, the WI was calculated in order to describe the color difference between the SPPS and Mucorpepsin coagulum. WI values are in the same range with those reported by [19] for cheese characterization. WI was lower for SPPS coagulum when compared to the commercial one ($P < 0.05$). A reduction in WI value revealed modification in coagulum matrix, such as increasing preteolysis and solubilization of colloidal calcium phosphate, directly reducing coagulum whiteness and increasing also pH value [20].

Regarding the viscosity evaluation, SPPS coagulum presented a significantly higher viscosity compared to that of Mucorpepsin one. This could be associated with the increase in total solid contents present in the final coagulum structure [21].

The pH values of SPPS and Mucorpepsin coagulum were both acidic varying from 5.16 (SPPS) to 5.57 (Mucorpepsin) (Table 3).

Table 3: *Physical properties of SPPS and Mucorpepsin coagulum.*

Enzyme / Proprieties	SPPS	Mucorpepsin
pH	5.16 ± 0.01^{a}	5.57 ± 0.03^{b}
L*	93.02 ± 0.11^{a}	92.61 ± 0.08^{a}
a*	-2.39 ± 0.04^{b}	-2.49 ± 0.03^{a}
b*	15.64 ±	14.27 ± 0.17^{a}
WI	0.07^{b}	49.79 ± 0.55^{b}
	46.11 ± 0.31^{a}	
Viscosity (mPas.s)	44 ± 1^{b}	40.67 ± 0.58^{a}

Different superscript letters (a-b) indicate significant differences according to t-test for two independent samples with at a significance level $P < 0.05$.

Besides, pH of Mucorpepsin coagulum was significantly higher than that of SPPS ($P < 0.05$). The differences in the colors are also revealed in Table 3. In this respect, the lightness reflected as the L* value was not significantly different in between the coagulum. The redness of the samples expressed as a* values was significantly higher for the SPPS one. As observed in the Table 3, the yellowness, b* values, was significantly lower for the Mucorpepsin coagulum. The index of whiteness was significantly higher for the Mucorpepsin coagulum (347.05) ($P < 0.05$). In addition, Table 3 illustrated also the viscosity of the two samples and showed that the SPPS coagulum presents a significantly higher viscosity (44 mPas.s) when compared to the Mucorpepsine one (40.67 mPas.s) ($P < 0.05$).

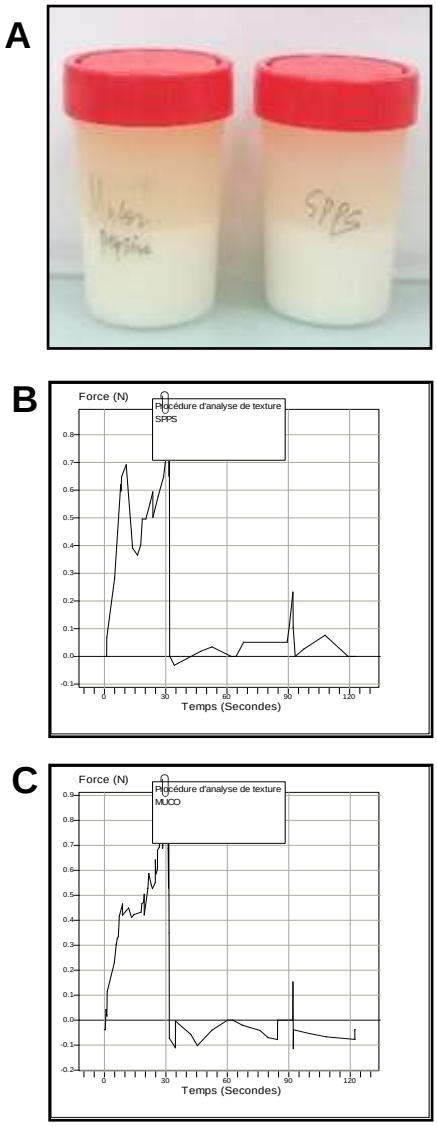

Figure 2. *Evaluation of the enzymatic performance proteases in milk coagulation* ***(A)*** *SPPS and Mucorpepsin enzymes. Texture analysis procedures of SPPS* ***(B)*** *and Mucorpepsin* ***(C)****.*

III.6. Cost Estimation

The protease production process is: submerged fermentation, centrifugation, and evaporation as presented in Fig. 3.

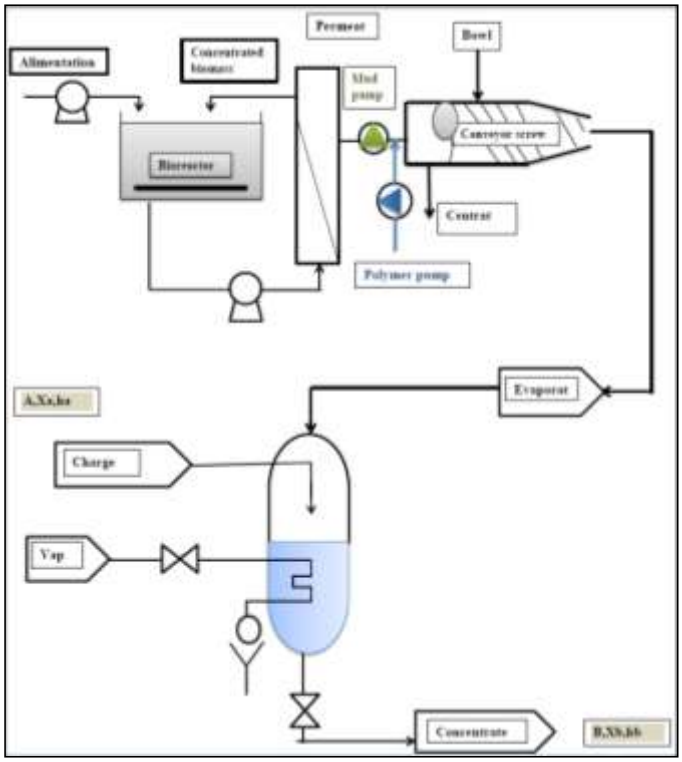

Figure 3. *Schematic diagram of SPPS production system.*

The cost estimated of the two production steps is 140.23 + 19 + 7 = 166.23 Tunisian Dinar (TND). This cost is detailed in Tables 4 and 5.

Table 4. *Direct and indirect costs of 70 liters enzyme production.*

Direct costs of 70 litre enzyme production (TND)	
Fluid	5.97
Steam	25
Air	8.86
Electricity	36,4
Instruments control	64
Production cost (TND/L)	140.23
Indirect costs of 70 litres enzyme production (TND)	
Engineering and supervision	5,400
Profit margin	12,240
Cost of return	17,640
Selling price (TND/kg)	7

Table 5. *Medium culture products for 100 liters of fermentation at UVRR-CBS (actually 70 liters).*

Medium composition	**Price (TND)**
Lentil flower (15 g/L)	5
Yeast extract (2 g/L)	14
Scale-up to 70 L	19

The evaporation cost is 1.77 TND for 70 L. So, the one liter production cost is17 TND. 100 mL are evaporated into 1 kg SPPS solid.SPPS can have a competitive price in comparison to commercial protease such as Mucorpepsin and Presura. The medium protease price is 5 or 6 Euros (€) that is to say 16 or 17 TND. Or, SPPS price is 7 TND which is refers to 2.2 €. So, we can say that SPPS is competitive even for the world market. The use of the computerized technical and economic assessment model developed in this project has assessed the financial interest in producing fungal proteases for use in cheese making.

IV. Conclusion

Fungi proteases have an important value in global enzyme market. These biocatalysts are of great economic importance, considering the multiple industrial applications in which they are involved. The possible application of these proteases in cheese formulation involves the development of an economical and reproducible production process. For this, the scale-up of the SPPS production was elaborated. Then, the determination of different step for the SPPS production to be ready to be used in cheese production industry. The physical characterization of SPPS and Mucorpepsin coagulum were studied. SPPS coagulum has excellent physical characteristics toward Mucorpepsin coagulum. According to the technico economic evaluation, the project of SPPS production for cheese making is profitable. It can have a competitive price in the market world. For perspective, we think to have a real start-up for the SPPS.

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Compliance with Ethical Standards

Competing Interests

Author Bassem Jaouadi was employed by the company Biotech ECOZYM Start-up at the Business Incubator at the CBS, Sfax, Tunisia. All other authors declare no competing interests.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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