Statistical optimization of an acid protease production by a local *Aspergillus niger* MH109542 using a medium based on decommissioned dates

Malika Benkahoul^{a*}, Amina Bramki^b, Aicha Belmessikh^a, Aicha Mechakra-Maza^a

^aUniversity of Mentouri Brothers-Constantine1. Faculty of Nature and Life Sciences, Department of Microbiology, Laboratory of Biology and Environment, P.O. Box,325 Ain El Bey Way, Constantine, Algeria

^bUniversity of Mentouri Brothers-Constantine1. Faculty of Nature and Life Sciences, Department of Microbiology, Laboratory of Mycology, Biotechnology and Microbial Activity, P.O. Box,325 Ain El Bey Way, Constantine, Algeria

Received 26th August 2019 / Accepted 24th January 2020

Abstract. The production of an acid protease by liquid fermentation is performed on a medium based on decommissioned dates by a local mold isolated from an extreme environment. The used mold is isolated from the thermal soil in Hammam Safsaf of Teleghma (Algeria). Phenotypic and molecular identification has shown that it is *Aspergillus niger*. This fungi strain exhibited exo-protease activity on milk agar. The fermentation medium based on decommissioned dates is enriched by other factors according to a statistical method; the plan of Plackett and Burman. This method (N = 8 experiments and N-1 factors) allowed the optimization of the enzyme production and the growth of the mold. The statistical analysis of the obtained results shows an increased enzyme activity (650.20U), in the presence of yeast extract and salts with probabilities $p = 0.239$ for the first and $p = 0.190$ for the second. Furthermore, the corn-steep-liquor ($p = 0.229$) and yeast extract ($p = 0.053$) present a significant effect on mold growth. In conclusion, the culture of *A. niger* on optimized medium gives good yields of biomass and proteolytic activity compared to the basal medium. These results are encouraging. In fact, the use of a cheap and available substrate such as decommissioned dates saves the cost price of fermentation on an industrial scale.

Keywords: *Aspergillus niger*, acid protease, decommissioned dates, liquid culture, Plackett and Burman matrices, thermal soil

INTRODUCTION

The Algerian date palm is much exploited; it represents the pivot of the oasis ecosystem through the number of existing palms, estimated at more than 8 million. According to statistics provided by the Ministry of Agriculture, the productive potential in 2006 was estimated at 500 000 tones. Dates are rich in sugars and present low levels of proteins (Vayalil 2012; Mkaouar & Kechaou, 2013; Benkahoul *et al.*, 2017). Despite this low content, the date proteins are qualitatively

balanced. The dates wastes described as "lost" are dates that are not consumed by man, either because of their weak taste qualities or because of their textures (too hard). The valorization of date's wastes made it possible to have products with the advantage of presenting interesting characteristics both organoleptically and nutritionally (Siboukeur *et al.*, 2013). Dates are rich in carbohydrates (mainly glucose and fructose) as well as a range of minerals and vitamins (Kamel,

*Author for correspondence: Malika Benkahoul, University of Mentouri Brothers-Constantine1. Faculty of Nature and Life Sciences, Department of Microbiology, Laboratory of Biology and Environment, P.O. Box,325 Ain El Bey Way, Constantine, Algeria. Email – benkahoul.malika@umc.edu.dz

1979; Nancib *et al.*, 2001) hence the interest of using them as a substrate in the fermentation process for enzymes production (Ait Kaki El-Hadef El-Okki *et al.*, 2016).

The use of both cheap and available agro-industrial residues as well as the optimization of nutritional and physicochemical conditions of microbial culture in enzymes production by bioprocesses is a good added value (Paranthaman *et al.*, 2009; Benkahoul *et al.*, 2017).

The global industrial demand for microbial enzymes is increasing from a year to another and this, to improve certain manufacturing processes such as food and textile industries (Kumar *et al.*, 2008a). To cope with this demand, particularly in biocatalysis field and based on the organic synthesis model "more respectful to the environment", many efforts have been made for the research of industrial interest enzymes. Special attention for enzymes coming from extremophilic microorganisms has been the subject of several studies. Indeed, extremophilic microorganisms have developed strategies to adapt to environmental constraints of their environment (temperature, pressure, salinity and pH). Enzymes that participate in these strategies will be no doubt of great interest to intervene in industrial processes working under high-temperature conditions to avoid contaminations (Gregoire *et al.*, 2009).

Proteases constitute the most important group of industrial enzymes with 40% to 60% of total enzyme sales, of which two-thirds are microbial (Gupta *et al.*, 2002; Garcia-Gomez *et al.*, 2009).

The optimization process is a central importance topic for industrial production. In the case of enzymes overproduction, manipulations of culture media represent the best alternative to genetic manipulations (Larpent and Sanglier, 1992).

The factorial experimental designs allow measuring the effect of several factors as well as the interactions between factors (Kammoun *et al.*, 2008). The most commonly used experimental designs are fractional factorial designs of Plackett and Burman (1946). This one has proved to be an efficient tool to rapidly determine the effect of different variables on the yield of a required product. It is used to minimize the number of screening variables for optimization studies.

These statistical models have been used to optimize enzymes production by yeast culture conditions (Bennamoun *et al.*, 2016) and molds (Belmessikh *et al.*, 2013; Sathishkumar *et al.*, 2015; Ait Kaki El-Hadef El-Okki *et al.*, 2016; Gasmi and Kitouni, 2016; Benkahoul *et al.*, 2017).

The main objective of this present study was to optimize the production of an acid protease from *Aspergillus niger* isolated from the thermal soil in Hammam Safsaf of Teleghma (Algeria), using agro-industrial residues (decommissioned dates) as the base medium.

MATERIALS AND METHODS

Biological material

Microorganisms that resist extreme conditions are highly sought-after by industries. The mold used in this study was isolated from the thermal soil in Teleghma region (located in the northeast of Algeria). After isolation and purification, the fungal strain was tested for its ability to produce exo-cellular proteases on milk agar. The presence of a transparent zone around the colony proves the mold's ability to hydrolyze milk proteins in the medium. The macroscopic and microscopic identification, based on colony aspect and the morphology of fructifications is confirmed by molecular analyzes based on the sequencing of ribosomal DNA ITS4 region. The DNA encoding for the 18S ribosomal RNAs of the isolate, extracted, and amplified is separated by agarose gel electrophoresis, and the resulting PCR products were sequenced, after that, the obtained sequence was subjected to a search for compatible sequences homology using the BLAST program (Blast Local Alignment Search Tool) on the site National Center for Biotechnology Information (NCBI) to search for the closest sequences filed in Genbank (<http://www.ncbi.nlm.gov/blast>: (Altschul *et al.*, 1997). After the treatment and cleaning of the nucleotide sequence with Chromas software (Larkin *et al.*, 2007), the phylogenetic tree was built with the Mega program Molecular Evolutionary Genetics Analysis program (MEGA) version 6 using the "Neighbor Joining method" (Saitou and Nei, 1987; Kumar *et al.*, 2008b).

Culture medium and fermentation conditions

The used of decommissioned dates are unfit for human consumption. After their recovery from BISKRA (TOLGA), Algeria and before using them, they are first dried in the shade, and then crushed. The basic culture medium was prepared using 20 g of decommissioned dates powder dissolved in 500 ml of distilled water. The suspension was centrifuged at 15000 g for 20 minutes.

The supernatant was diluted with 0.1 phosphate buffer at pH 5, in order to give two different concentrations: 2% and 5%, respectively. The medium is enriched according to the experimental plan (Table 1) and then distributed in 250 ml Erlenmeyer flasks at a rate of 50 ml per flask. Before sterilization at 115°C for 20 min, the pH of each Erlenmeyer was adjusted. It was inoculated with 10^4 spores / ml, then homogenized and incubated at 30°C for 3 days under the agitation of 220 rpm by an orbital incubator shaker (Edison NJ. USA). The composition of the production medium in each Erlenmeyer varies according to the experimental design. After the three days of incubation, the protease activity and biomass were measured.

Statistical method

Several factors can influence a given process. But the factors with a signification at or above 70% are accepted and considered with an effect on the response. Optimizing a process consists of identifying factors that have significant effects on the process. The Plackett-Burman statistical model is a fractional factorial model. It is used to evaluate the effect of different factors in many fermentation processes. It is utilized for optimization by screening for X_i factors that can be susceptible to influence the Y response (Lewis *et al.*, 1999). Each factor can take two levels (lower (-1) or higher (+1)) (Table 2). A matrix of 7 factors (5 real factors and 2 unreal factors or errors) and 8 experiments were used (Table 1). To determine whether the factors have a significant effect or not; a simple polynomial equation of the first order is proposed: $Y = \beta_0 + \sum \beta_i X_i$.

Statistical analysis

At the end of each experiment, the effect of each factor on the production of enzyme and biomass is estimated as the difference between the average

of the response values observed at the higher level (+) and the average of the response values observed at the lower level (-) according to the following mathematical expression:

$$E_i = \frac{\sum \text{Responses at the level (+)}}{\text{Number of values (+)}} - \frac{\sum \text{Responses at the level (-)}}{\text{Number of values (-)}}$$

E_i : effect of each factor.

The significance of each effect is determined by the Student's test (t).

$$t = \frac{\text{Effect}}{E \times S}$$

The calculations are performed by the "Minitab 16" software. All variables with a probability $p > 0.3$, meaning a signification less than 70% are rejected. Enzyme and biomass production results are presented in the form of a first-order polynomial equation with only the explanatory variables with a significant positive or negative effect.

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + e$$

β_0 : Constant

y : The experimental response;

X_i : Explanatory variables;

β_i : The coefficients of the explanatory variables;

e : The average of the experimental errors.

The performed assays

The biomass

The biomass is separated from the culture medium by a simple filtration on Whatman paper No. 1. The wet biomass is dried in an oven at $103^\circ\text{C} \pm 2^\circ\text{C}$ and then weighed several times until the stability of weight called dry weight.

Protease activity

The acid protease activity is measured at pH 5 on casein according to Anson's method, (1938) modified and described by Mechakra *et al.* (1999) and Benkahoul *et al.* (2017). The nonprotein nitrogen compounds (tyrosine) produced are assayed by colorimetry, according to Folin and Ciocalteu (1927) referring to a calibration curve of tyrosine. The proteolytic activity unit corresponds

to 1 µg of released tyrosine per hour and per ml of culture medium.

Table 1. Design matrix of Plackett-Burman design.

Run order	Factors						
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇
1	+1	+1	+1	-1	+1	-1	-1
2	-1	+1	+1	+1	-1	+1	-1
3	-1	-1	+1	+1	+1	-1	+1
4	+1	-1	-1	+1	+1	+1	-1
5	-1	+1	-1	-1	+1	+1	+1
6	+1	-1	+1	-1	-1	+1	+1
7	+1	+1	-1	+1	-1	-1	+1
8	-1	-1	-1	-1	-1	-1	-1

Table 2. Minimum and maximum ranges of the different factors studied in the Plackett–Burman design.

Factors	Lower level (-1)	Higher level (+1)
X ₁ : Corn-steep liquor (ml/L)	0	2%
X ₂ : <i>Galactites tomentosa</i> flower petals (g/L)	0	2%
X ₃ : error	-	-
X ₄ : medium concentration in decommissioned dates (g/L)	2%	5%
X ₅ : error	-	-
X ₆ : Yeast extract (g/L)	0	2%
X ₇ : Salts		
MnSO ₄	0	0.5 g / L
ZnSO ₄	0	0.5 g / L
FeSO ₄	0	0.5 g / L
MgSO ₄	0	0.5 g / L

RESULTS

Identification of the selected strain

Based on the keys of fungal genera determination namely cultural characteristics and microscopic morphology (size, shape and conidia arrangement on the conidiophore) established by Botton *et al.* (1990) and Chabasse *et al.* (2002), it turned out that the selected fungal strain belonged to *Aspergillus* genus. The phenotypic identification was confirmed by molecular analysis based on the sequencing of 18S ribosomal DNA. After the extraction and amplification of DNA, the obtained PCR products were sequenced; the sequence was compared to those of other

microorganisms recorded at the Genbank database. This proposes a sequence that presents the best bit score and identity percentage with ours (Table 3). These sequences showed a similarity of 99% with many *A. niger* strains.

Table 3. Confrontation and bio molecular correspondence with GenBank.

Isolate	Strain proposed by GenBank	Score	E-value	Identity
T5	<i>Aspergillus niger</i>	834	0.0	99%

From the obtained sequence, a phylogenetic study was carried out. Sequence alignments are made by ClustalW. The tree is constructed from Neighbor-joining using the bootstrap test (numbers on the branches) of 1000 replications (Figure 1). The sequencing of rRNA gene and the alignment with sequences extracted from Genbank confirm that the T5 isolate belongs to *Aspergillus niger* species. The obtained sequence was deposited in the Genbank database of NCBI under the accession number MH109542.

Figure 1. Tree using the distances method (Neighbor-joining) constructed from the analysis of 18s rRNA sequences using Mega6 software showing the phylogenetic position of T5 isolate among the representative species of *Aspergillus* genus.

Selection of protease production factors and mold growth using Plackett and Burman design

Figure 2 and Figure 3 showed results of the factors effects on both responses, protease activity and biomass obtained by statistical analysis (Tables 4 and 5).

Effects of tested factors on protease production

Seven variables supposed to affect the acid protease production are evaluated by eight experiments according to Plackett and Burman

statistical design. Figure 3 showed the responses obtained in terms of estimated protease activity by colorimetric method for each experiment. It shows that the maximum protease activity (650.20 U) is found in trial no. 5, while the lowest activity is found in trial no. 8, (the basal medium). The responses are evaluated statistically (Table 4). Variables with p less than or equal to 0.3 and a confidence level greater than or equal to 70% are considered to have a significant effect on the response. The most significant variables positively affecting protease production by *A. niger* MH109542 (Table 4) are salts ($p = 0.190$), with a confidence level of 81% and yeast extract ($p = 0.239$), with a confidence level of 76%. The measured probabilities in the presence of *corn-steep-liquor* and flower petals as well as the medium concentration in decommissioned dates are $p = 0.912$, $p = 0.510$ and $p = 0.523$ respectively; values greater than 0.3. Therefore, these factors have no significant effect on protease production (Table 4).

Figure 2. Experimental results of biomass of *A. niger* MH109542 for 72 hours incubation.

Figure 3. Experimental results of protease produced by *A. niger* MH109542 for 72 hours incubation.

Effects of tested factors on biomass production

The results of the different factors effects on biomass are represented in Figure 2 which showed better growth in trial no. 4, while the lowest growth is obtained in trial no. 8 (basal medium).

The statistical results in Table 5 show that the presence of corn-steep liquor in the composition of culture medium increases the growth of *A. niger* and present a probability $p = 0.229$, that means a positive significance equal to 77.6%. On the other hand, yeast extract is highly significant on biomass ($p = 0.053$), that means 94.8% of significance. The presence of dates with a concentration of 5% in the culture medium present a positive effect but, it is not significant on mycelial growth ($p = 0.330$). Despite their high protein content, the presence of *Galactites tomentosa* flower petals presents an insignificant effect on biomass ($p = 0.520$). It is the same for salts ($p = 0.396$).

Expression of responses in the form of equations

From the presented results in Table 4 and Table 5, and after exclusion of insignificant factors (less than 70% of significance) on responses, protease activity and biomass, the responses can be written according to the reduced polynomial equation (1):

Protease production:

$$Y = 217 + 103 X_6 + 122 X_7 \quad (2)$$

X_6 is the yeast extract, X_7 is the salts.

Biomass production

$$Y = 0,750 + 0,157 X_1 + 0,385 X_6 \quad (3)$$

X_1 represent the *corn-steep-liquor* and X_6 the yeast extract.

DISCUSSION

In the Middle East, the use of *Aspergillus* genus molds in agri-food interest enzymes production date for over 2000 years, whereby Koji, which is a complex enzyme, has been used in the production of soy sauce, miso and sake. In Europe, the *Aspergillus sp.* enzymes were used from the beginning of the 20th century in bakery and brewery (Barbesgaard *et al.*, 1992).

Table 4. The effects of the explicative factors on the protease activity.

Factors	Estimate of coefficients	Test value (t)	p value	Significance of probability
Corn-steep liquor	7.8	0.13	0.912	-
<i>Galactites tomentosa</i> flower petals	49.50	0.79	0.510	-
Error	-78.6	-1.01	0.359	-
Medium concentration in decommissioned dates	- 47.9	-0.77	0.523	-
Error	39.8	0.51	0.631	-
Yeast extract	103.7	1.66	0.239	76%
Salts	121.68	1.95	0.190	81%

Table 5. The effects of the explicative factors on the biomass.

Factors	Estimate of coefficients	Test value (t)	p value	Significance of probability
Corn-steep liquor	0.157	1.71	0.229	77.6%
<i>Galactites tomentosa</i> flower petals	-0.0713	-0.77	0.520	-
Error	-0.006	-0.03	0.976	-
Medium concentration in decommissioned dates	0.118	1.28	0.330	-
Error	0.130	0.65	0.546	-
Yeast extract	0.385	4.18	0.053	94.8%
Salts	-0.0987	-1.07	0.396	-

The identification of the used strain in this study not only makes it possible to list it but also to know its properties, thanks to its genetic background and the ability to synthesize certain metabolites. This strain (*Aspergillus niger* MH109542) has shown its ability to produce exo-cellular proteases. In fact, the formation of a transparent halo around the colonies, resulting from the degradation of milk proteins in the medium, is observed from the first 24h of incubation. It must also be mentioned the rapid growth of the mold characterized by a total invasion of the culture medium within four days.

The optimization of protease production and mold growth is achieved by a statistical method using fractional factorial designs of Plackett and Burman (1946). The conventional approach consists of varying successively a single factor while maintaining the other constants. As a result, the experiments are long and costly. In addition, when a large number of variables are considered, the method does not take into account the interactions between factors (Kammoun *et al.*, 2008). The use of factorial experiment designs not only allows measuring the effect of several factors but also their interactions (Box and Wilsson, 1951). The matrices of Plackett and Burman are widely used in the selection of culture media components.

The maximum acid protease activity (650.20 U) was found in trial no. 5. This result is higher than 181,13U, obtained by *A. niger* grown in media using two cheap and fermentation substrates, the hulled grain of wheat and shrimp peptone (Siala *et al.*, 2012). However, Soares de Castro and al. (2016) tested the production of proteases by *A. niger* LBA02 under solid-state. They used wheat bran as substrate and showed that the highest protease activity was 186.42U.

The fermentation results show an improvement in the production of acid protease in the presence of mineral salts that exert a positive effect with a threshold of 81%. Minerals promote microbial growth and therefore, increase metabolites production yields as reported by Tunga *et al.* (1998).

Protease production may also be affected by nitrogen sources (Scriban, 1993). Despite its high nitrogen content, corn-steep-liquor has no significant effect on the production of acid protease, while the yeast extract significantly affects the enzyme production by *Aspergillus niger* MH109542. The improvement of the acid protease production is related to the richness of yeast extract in proteins and amino acids. High protease activity is observed when the waste from food industries medium was supplemented with yeast extract (Hernandez *et al.*, 2006). In fact,

Golla (2018) reports that the production of acid protease by *Aspergillus sp* is much better with organic nitrogen sources than inorganic sources. For this, several studies mention the importance of yeast extract as a nitrogen source (Teodoro and Martins, 2000); this one stimulates the production of the protease by several microbial species (Phadatare *et al.*, 1993; Ashour *et al.*, 1996; Badhe *et al.*, 2016).

The statistical analysis of *Aspergillus niger* MH109542 growth results shows a significant effect exerted by both *corn-steep-liquor* and yeast extract. The literature reports variable results concerning the composition of *corn-steep liquor* according to its source (Hull *et al.*, 1996; Gao and Yuan, 2011) but all the references present it as a by-product encompassing a wide variety of amino acids (Xiao *et al.*, 2013) using Plackett and Burman's design, Djekrif-Dakhmouche *et al.* (2006) also note an increase in *A. niger* growth with a significance of 90%. The supplementation with a nitrogen source allows balancing the culture medium; it constitutes 1 to 15% of cells weight. The presence of the yeast extract in the culture medium had a highly significant effect on the growth of *A. niger* MH109542. Therefore, this factor is a good source of nitrogen and growth factors. Indeed, it is used in the majority of culture media for its valuable amino acid content (Scriban, 1993). The presence of mineral salts in the culture medium has no significant effect on biomass. The non-negligible composition of dates in the mineral matter (Kamel 1979; Nancib *et al.*, 2001; Benkahoul *et al.*, 2017) seems sufficient for the mold growth. Therefore, mineral supplementation is not necessary. In fact, the date powder has high levels of potassium, magnesium and average levels in calcium, phosphorus and iron (Mkaouar and Kechaou, 2013).

The results reveal that an increase in basal medium concentration in decommissioned dates has no significant effect on either biomass or protease production. The richness of dates in carbohydrate gives them a high C/N ratio (Ait Kaki El-Hadef El-Okki *et al.*, 2016), which makes this substrate a good source of carbon, this one can cover the needs of microbial growth (Kamel, 1979; Nancib *et al.*, 2001). In this case, a basal medium concentration of 2% in dates seems sufficient for the development of *Aspergillus niger* MH109542.

The obtained fermentations results show that the used decommissioned dates in the composition of basal medium constitute a good source of carbon and minerals for the growth of *Aspergillus niger* MH109542. The used nitrogen and carbon sources are suitable for both microbial growth and acid protease production. These same results showed that *Aspergillus niger* MH109542 locally isolated from soil close to thermal waters, can be used successfully for producing an acid protease on an optimized and cheap medium.

CONCLUSION

The present study examined the optimization of an acid protease production by a filamentous mold locally isolated from an extreme environment. It is *Aspergillus niger* MH109542 using as basic medium decommissioned dates. These optimized media are composed of natural and inexpensive substrates leading to a considerable reduction in the cost of production, as required for industrial enzymes production. Our study leads us to conclude that the design of Plackett and Burman is an ideal one for factor screening and has allowed us to select three factors: *corn-steep liquor* which shows a positive effect on biomass ($p = 0.229$), yeast extract which represents a source of nitrogen for the growth of the mold ($p = 0.053$) on the one hand, and for the production of the protease production ($p = 0.239$) on the other hand and salts ($p = 0.190$) which are essential for the stimulation of the production of protease.

REFERENCES

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25 (17): 3389-3402.
- Ashour, S. A., El-Shore, H. M., Metwally, M., & Habib, S. A. 1996. Fungal fermentation of Whey incorporated with certain supplements for the production of protease. *Microbios* 86 (346): 59-69.
- Ait Kaki El-Hadef El-Okki, A., Gagaoua, M., Bennamoun, L., Djekrif, S., Hafid, K., El-Hadef El-Okki, M., & Meraihi, Z. 2016. Statistical Optimization of thermostable α -amylase production by a newly isolated *Rhizopus oryzae* strain FSIS4 using decommissioned dates. *Waste Biomass Valor* 8 (6): 2017-2027.

- Anson, M. 1938. Estimation of pepsin, papain and cathepsin with hemoglobin. *Journal of General Physiology* 22: 79-89.
- Badhe, P., Joshi, M., & Adivarekar, R. 2016. Optimized production of extracellular proteases by *Bacillus subtilis* from degraded abattoir waste. *Journal of BioScience and Biotechnology* 5(1): 29-36.
- Belmessikh, A., Boukhalfa, H., Mechakra-Maza, A., Gheribi-Aoulmi, Z., & Amrane, A. 2013. Statistical optimization of culture medium for neutral protease production by *Aspergillus oryzae*. Comparative study between solid and submerged fermentations on tomato pomace. *Journal of the Taiwan Institute of Chemical Engineers* 44: 377-385.
- Barbesgaard, P., Heldt Hansen, H. P., & Diderichsen, B. 1992: On the safety of *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* 36(5): 569-572
- Benkahoul, M., Belmessikh, A., Boukhalfa, H., & Mechakra-Maza, A. 2017. Optimisation à l'aide d'un plan d'expériences de la production d'une protéase fongique sur milieu à base de déchets agro-industriels. *D S T - N°75*.
- Bennamoun, L., Hilgsmann, S., Dakhmouche, S., Ait-Kaki, A., Labbani, F-Z. K., Nouadri, T., Meraihi, Z., Turchetti, B., Buzzini, P., & Thonart, P. 2016. Production and properties of a thermostable, pH—stable exo-polygalacturonase using *Aureobasidium pullulans* isolated from Saharan soil of Algeria grown on tomato pomace. *Foods* 5(4): 72.
- Botton, B., Bretton, A., Fever, M., Gautier, S., Guy, P. H., Larpent, J. P., Reymond, P., Sanglier J-J., Vayssier, Y., & Veau, P. 1990. Moisissures utiles et nuisibles: importance industrielle. Masson., éd. Paris.
- Box, G. E. & Wilson, K. G. 1951. On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society* 13: 1-45.
- Chabasse, D., Bouchara, J. P., Gentile, L., Brun, S., Cimon, B., & Penn, P. 2002. Les moisissures d'intérêt médical. Bioforma., éd. Paris. 160p.
- Djekrif-Dakhmouche, S., Gheribi-Aoulmi, Z., Meraihi, Z., & Bennamoun, L. 2006. Application of a statistical design to the optimization of culture medium for α -amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder.
- Folin, O. & Ciocalteu, V. 1927. On tyrosine and tryptophane determination in protein. *Journal of Biochemistry* 73: 627.
- Gao, Y. & Yuan, Y.J. 2011. Comprehensive quality evaluation of corn steep liquor in 2-keto-L-gulonate fermentation. *Journal of Agricultural and Food Chemistry* 59(18): 9845-9853.
- García-Gómez, M. J., Huerta-Ochoa, S., Loera-Corral, O., & Prado-Barragan, L. A. 2009. Advantages of proteolytic extract by *Aspergillus oryzae* from fish flour over a commercial proteolytic preparation. *Food Chemistry* 112: 604-608.
- Gasmi, M. & Kitouni, M. 2016. Optimization of chitinase production by a new *Streptomyces griseorubens* C9 isolate using response surface methodology. *Annals of Microbiology* 67(2): 175-183.
- Golla, N. 2018. Production, optimization and partial purification of acid protease from *Aspergillus* spp. isolated from soil contaminated with abattoir waste. *BioTechnology An Indian Journal* 14(4).
- Gregoire, P., Fardeau, M.L., Guaso, S., Bouanane, A., Michotey, V., Bonin, P., Dubourg, K., Cambar, J., & Ollivier, B. 2009. Les micro-organismes de l'extrême. *Press Therm Climat* 146: 49-61.
- Gupta, R., Beg, Q.K., Khan, S., & Chahuan, B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology* 60: 381-395.
- Hernandez, M. S., Rodriguez, M. R., Guerra, N. P., & Rosés, R. P. 2006. Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. *Journal of Food Engineering* 73: 93-100.
- Hull, S. R., Yang, B. Y., Venzke, D., Kulhavy, K., & Montgomery, R. 1996. Composition of corn steep water during steeping. *Journal of Agriculture and Food Chemistry* 44(7): 1857-1863.
- Kamel, B. S. 1979. Dates as a potential substrate for single cell protein production. *Enzyme and Microbial Technology* 1(3): 180-182.
- Kammoun, R., Naili, B., & Bejar, S. 2008. Application of a statistical design to the optimization of parameters and culture medium for α -amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product). *Bioresource Technology* 99(13): 5602-5609.
- Kumar, D., Savitri, Thakur, N., Verma, R., & Bhalla, T. C. 2008a. Microbial proteases and application as laundry detergent additive. *Research Journal of Microbiology* 3(12): 661-672.
- Kumar, S., Nei, M., Dudley, J., & Tamura, K. 2008b. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* 9(4): 299-306.
- Larpent-Gourgau, M., Sanglier. 1992. Biotechnologies. Principes et méthodes.
- Larkin, M. A., Blackshields, G., Brown, N. P., Cenna, R., McGettigan, P. A., & McWilliam, H. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947-2948.
- Lewis, G., Mathieu, D., & Phan-Tan-Luu, R. 1999. Pharmaceutical Experimental Design, Marcel Dekker, ed. New York, pp. 498.
- Mkaouer, S. & Kechaou, N. 2013. Valorisation des écarts de triage de dattes par séchage pour l'obtention d'une poudre pour alimentation animale. *Dechets sciences et techniques* 63: 26-30.
- Mechakra, A., Auberger, B., Remeuf, F., & Lenoir, J. 1999. Optimisation d'un milieu de culture pour la production d'enzymes protéolytiques acides par *Penicillium camemberti*. *Sciences de Aliments* 19: 663-75.
- Nancib, N., Nancib, A., Boudjelal, A., Benslimane, C., Blanchard, F., & Boudrant, J. 2001. The effect of supplementation by different nitrogen sources on the production of lactic acid from date juice by *Lactobacillus casei* subsp. *rhamnosus*. *Bioresource Technology* 78(2):149-153.
- Paranthaman, R., Alagusundaram, K., & Indhumathi, J. 2009. Production of protease from rice mill wastes by *Aspergillus niger* in solid state fermentation. *World Journal of Agricultural Sciences* 5(3): 308-312.
- Phadatar, S. U., Deshpande, V. V., & Srinivasan M. C. 1993. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20), enzyme production and compatibility with commercial detergents. *Enzyme and Microbial Technology* 15(1): 72-76.
- Plackett, K. L. & Burman, J. P. 1946. The design of optimum multifactorial experiments. *Biometrika* 33:305-325.
- Saitou, N. & Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.
- Sathishkumar, R., Ananthan, G., & Arun, J. 2015. Production, purification and characterization of alkaline protease by ascidian associated *Bacillus subtilis* GA CAS8 using agricultural wastes. *Biocatalysis and Agricultural Biotechnology* 4(2).
- Scriban, R. 1993. Biotechnologie, 4^e édition. (pp. 90-717). Techniques et documentations. Lavoisier
- Siala, R., Frikha, F., Mhamdi, S., Nasri, M., & Sellami Kamoun, A. 2012. Optimization of acid protease production by *Aspergillus niger* I1 on shrimp peptone using statistical experimental design. *The Scientific World Journal* (3): 1-11.
- Siboukeur, A., Mimouni, Y., Hafiane A., & Siboukeur, O. 2013. Valorization of dates (cultivar "Ghars") by technological process. *Acta Horticulturae* 994: 57-261.

- Soares de Castro, R. J., Herculano Soares, M., Reolon Mangabeira Albernaz, J., & Sato, H. 2016. Biochemical characterization of solvent, salt, surfactant and oxidizing agent tolerant proteases from *Aspergillus niger* produced in different agro industrial wastes. *Biocatalysis and Agricultural Biotechnology* 5: 94–98.
- Teodoro, C. E. de S & Martins, M. L. L. 2000. Culture conditions for the production of thermostable amylase by *Bacillus* sp. *Brazilian Journal of Microbiology* 31 (4).
- Tunga, R., Banerjee, R., & Bhattacharya, B. C. 1998. Optimizing some factors affecting protease prooduction under solid-state fermentation. *Bioprocess Engineering* 19: 187-190
- Vayalil, P. K. 2012. Date fruits (*Phoenix dactylifera* Linn): an emerging medicinal food. *Critical Reviews in Food Science and Nutrition* 52: 249-271.
- Xiao, X., Hou, Y., Liu, Y. Liu, Y. Zhao, H., Dong, L., Du, J., Wang, Y., Bai, G., & Luo, G. 2013. Classification and analysis of corn steep liquor by UPLC/Q-TOFMS and HPLC. *Talanta* 107: 344-348.