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Biosynthesis of pectinases from free and immobilized *Aspergillus niger* and their application in the extraction of essential oil

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Abstract

The food processing industry is an advanced sector in strong evolution, turned to innovation and research and in which, the use of enzymatic processes takes center stage. In that capacity, pectinases is mainly used to clarify juices and to augment the output of extraction. In this sense, the present study was aimed at the improvement of output in essential oils of the rosemary (*Rosmarinus officinalis*) by the use of pectinases extracted from the free and immobilized *Aspergillus niger* EF97 strain. The preparation of pectinases enzymes was got from the culture medium supernatant of *Aspergillus niger* strain EF97 grown on pectin as a carbon source. Controlled parametres (concentration in pectin, temperature, pH and agitation of incubation) are optimized by the experimental planning method. The free cells in culture produce three pectinolytic enzymes; polygalacturonase (PG), pectinesterase (PE) and pectinlyase (PL). However, the mycelium entrapped in the calcium alginate matrix synthesizes only the polygalacturonase (PG) but with a high specific activity. The effect of enzymatic treatment by free and immobilized cells exhibited improvements in the yield of essential oils (lower energy cost and reduced treatment time). The yield of essential oils increased by 17% and the rate of hydrodistillation of the rosemary biomass treated with the enzyme preparation was faster (40%).

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Introduction

Aspergillus niger is an imperfect ascomycete fungus (Class: Deuteromycetes), belonging to the Aspergillaceae family (Dijksterhuis J. Eandal., 2013). It is grown on a wide range of substrates and controls many strains or subspecies. It is a mold that is widely encountered in industrial microbiology (Santé Canada, 2017) to produce enzymes (biocatalysts) of great interest such as pectinases, amylases and proteases. Pectinases are a complex enzymatic system that catalyzes the total hydrolysis of pectin (Bayoumi *et al.,* 2008). Their applications in the food industry predominate, such as the maceration and liquefaction of fruits as well as the extraction and clarification of their juices. (Rogerson and *al.,* 2000; Hoa *et al.,* 2013; Pinheiro *et al.,* 2017).

Many studies have been carried out to select competent strains in the production of pectinases and optimization of the parameters of their synthesis (Herron and Benen, 2000; Martos *et al.,* 2009; Khairnar Y. *et al.,* 2009; Nazneen A. *et al.,* 2012; Barman S *et al.,* 2015). Current trends are aimed at improving the productivity of these enzymes by stimulating cell metabolism via innovative technologies. Several authors state that the technique of immobilization of whole cells gives a better enzymatic yield than that of free cells (Silva *et al.,* 2003; Ibrahim *et al.,* 2004, Kassim. 2012).

Many studies were published on these themes, that it is linked to immobilization techniques or to the used support. However, at present, very few industries use it as one of the most innovative techniques in the optimization of agri-food bioprocesses. This work is part of this path. The target industry is the extraction of essential oils of rosemary (*Rosmarinus officinalis*) via an enzymatic process using pectinases, recognized by their very high pectinolytic power. Indeed, this group of enzymes of interest, extracted from free and immobilized *Aspergillus niger* EF97 cells, would be the key tool of a better optimization, as well of the yield as of the quality of the essential oils coming from this this emblematic plant of Moroccan traditional medicine, while reducing the cost of this bioprocess.

Material and methods

Distillation of essential oils

Essential oils, these volatile and odorous substances highly prized by the world of cosmetics and aromatherapy among others, are unfortunately present at very small quantities in plants, with a percentage that does not generally exceed 1%. Thus, the techniques of their extraction must be more and more innovative and satisfactory. In our investigation, we opted for hydro distillation, the technique, not only the oldest may also, the simplest and most widespread. During this process, the raw material is immersed directly in the boiling water, the evaporation of water in the still is carried out by direct heating (Eloutassi, 2004, Ouis, 2015). The vapors formed are condensed by a water flow refrigeration system.

Coupling Enzyme-Distillation

Strains and culture conditions

The *Aspergillus niger* EF97 strain with the ability to grow on the lignocellulosic waste powder was isolated in a preliminary microbiological study. His taxonomic affiliation was verified by the method described by (Robert *and al.* (1981).

The culture medium consists of pectin, the concentration of which has been optimized by the experimental planning method (Carlson and Nordahl, 1993). The optimum conditions used are 1.5% pectin. The environment is complemented by $(NH_4)^2$ SO4 (0.3%) p/v, KH_2PO_4 (0.1%) p/v and yeast extract (0.1%) p/v. The effective volume of the culture medium is 100ml in a 250ml, the incubation temperature is 30°C. And the agitation of 50 rev/min. Samples of the culture medium were taken to directly evaluate the specific activities of pectinase enzymes. A spore suspension (109 spores per ml) was used as an inoculum for both free and immobilized cell culture techniques. Iimmobilization was done by the method described by Jamai *et al.,* (2001). Cell growth was determined by measuring free total CO_2 in the culture medium.

Enzyme-plant coupling effect

The effect of the pectinolytic enzymes on the yield of essential oils extracted from the leaves of rosemary was evaluated by adding the concentrate from

ultrafiltration (amicon cell 52, diaflo membrane pm 10) of the supernatant of the culture medium of *Aspergillus niger* EF97 containing pectin as inducer of pectinolytic enzymes. 20ml of the concentrate was added to 100 g of rosemary grinding. The controlled parameters (time, temperature, pH and agitation) are optimized by the experimental planning method (Carlson and Nordahl, 1993). The essential oil yield was measured after hydrodistillation.

Catalytic activity of pectinolytic enzymes

Polygalacturonase activity (PG, EC 3.2.1.15) was analyzed by measuring the amount of reducing sugars released according to Miller (1969). In the pectin solution (50mM citrate buffer). One unit of PG activity (U/ml) is defined as the amount of enzymes at pH 5 which catalyzes the formation of 1 µmol of galacturonic acid per hour per ml. Pectinesterase activity (PE, EC 3.1.1.11) was determined by the method of Minussi and al. (1998) and Rigal (2001). One unit of PE activity (U/ml) is defined as the amount of enzyme which liberates 1 µmol of carboxyl groups per ml per min. Pectinlyase activity (PL, EC 4.2.2.10) was calculated by the method of Albersheim (1966). One unit of enzymatic activity was defined as the amount of enzyme that catalyzes an increase in absorbance of 0.555 at 235 nm for 1 min, at 25 ° C and pH 6.5.

Analytical methods

The substrate used in the assay of the three enzymes is citrus polygalacturonic acid: (minimum 85%). The colorimetric assay of proteins made according to the modified Lowry method (Tan *et al.,* 1984) with crystalline bovine albumin as standard. The reducing sugars were determined by the dinitrosalicylic acid (DNS) method according to Miller (1969). CO2 was measured by the gravimetric method (Pochon *et al.,* 1962).

Results and discussion

Yield in essential oils according to the distillation method

The method of getting of essential oils intervenes in a decisive way in output and chemical composition of got extracts. The distillation, discovered by the big doctor Abu Ali Ibn Sina, commonly known as steam training, remains at the moment the most adopted by the industry working in the extraction of essential oils. It consists of recovering the essential oils contained in the plants by water vapor, the combination of steam and heat explodes the cellular structure of the plant material and releases its essential oil. The water vapor causes the volatile compounds to the refrigerant, they then return to the liquid state. Two liquid phases are then obtained: the essential oil and the water. Decanted water called hydrolat remains very fragrant (Mazni I., 2007). Volatile products from distillation are easily analyzed by separation techniques requiring a relatively simple technology and therefore a lower cost (Eloutassi, 2004).

In preliminary tests, we opted for a steam drive system through a separate generator (Fig. 1). The yields of essential oils were unsatisfactory because, in the best case, they did not exceed 0.83 g per 100 g of the plant. Also, it should be noted, the multiple disturbances that hindered the control of evaporation in the boiler and liquefaction in the condenser.

While in more advanced tests, hydrodistillation has resulted in progressive yields with increasing dimensions of distillers to reach a value of 1.2 g of essential oils per 100 g of the plant (Fig. 1). This allowed us to further optimize the geometrical shape and the thermodynamic conditions of the automated distillation system to save the heat energy dissipated in the heating, to also identify the amount of water used in the condensation and finally to limit the losses of essential oils by working in a closed system (this is a separate work).

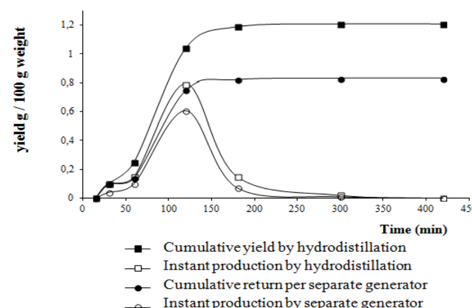

Fig. 1. Yield in essential oils according to the distillation method.

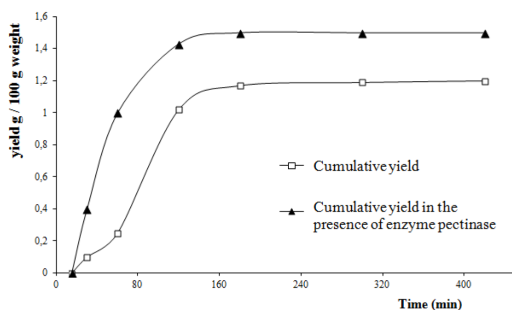

Fig. 2. Effect of pectinase on the production of essential oils.

Effect of pectinases-essential oils

In order to control and improve the local production of essential oils we carried out batch kinetics to determine the maximum yield and the total yield in the presence and absence of pectinase enzymes (Fig. 2).

In Fig. 2, considerable variations have been obtained from the cumulative production of essential oils in the presence and absence of the pectinase enzymes as a function of the duration of the distillation. The presence of pectinolytic enzymes reduced the distillation time and increased the weight yield of essential oils compared to the rosemary biomass).

The pectic substances include a complex set of polysaccharides characterized by a high content of polygalacturonic acid (α-1-4 linked galacturonic acid units, some of the carboxylic positions of which are methoxylated). These substances play a determining role in the physicochemical properties of the cell wall. This polymer is degraded thanks to the intervention of 3 types of enzymes: Pectinelyase, Polygalacturonase and Pectin esterase.

(Rigal, 2001). The action of the pectolytic activities allows the lysis of the rosemary cell wall (synergistic effect) and makes it possible to increase the extraction of certain constituents contained in the cell wall, such as phenolic compounds (extraction of color) and the precursors aromas. Vries and Visser (2001). This lysis increases the amount of essential oils that are very rich in aromas, tannins and coloring substances (anthocyanins). The tannin-anthocyanin combination gives stability to the color (Selmaoui *et al.*, 2017).

This activity improved yields (17%) and made the operation as simple as profitable. Thus, it is important to underline the multiple virtues of such a bioprocess in the valorization of the vegetable biomass of rosemary and thus to incite the industrialists of the sector to highlight these results through pilot tests.

Biosynthesis of pectinases

After the assays, the enzymatic units used in the extraction of essential oils of rosemary are summarized in Table 1. The concentrate contains respectively three types of pectinolytic activity: polygalacturonase (PG), pectinesterase (PE) and pectinlyase (PL). These amounts of pectinolytic enzymes expressed in enzymatic units, obtained after concentration of the supernatant of the culture medium by ultrafiltration are similar to those used in agri-food industries.

Table 1. Pectinolytic enzymes produced by the strain *Aspergillus niger* EF97.

Pectinolytic activity	Supernatant	Concentrated
Polygalacturonase PG (U/ml)	9.8	92
Pectinesterase PE (U/ml)	2.5	21
Pectinlyase PL (U/ml)	1.9	17

The results of pectinase production by the *Aspergillus niger* strain and the growth rate of the latter are summarized in Table 2. Free cells produce three types of pectinolytic activities (PG, PE and PL), however the mycelium entrapped in the alginate matrix (Fig. 3) synthesizes only polygalacturonase (PG) but with a higher specific activity.

Immobilized cells showed significant inhibition of the pectinesterase (PE) and pectinlyase (PL) enzymes. The matrix would therefore influence the expression of genes involved in the synthesis of pectinolytic enzymes: PE and PL. That said, the phenomenon of cell activity changes after immobilization remains poorly determined. The potential causes of these changes would be largely due to the effects of the microscopic environment, such as cell-cell and cell-matrix (alginate) contacts.

In addition, there is the effect of the diffusion of oxygen and nutrients to the cells on the one hand, and CO2 and metabolic products to the outside of cells on the other hand. So immobilization affects cell growth and the spectrum of pectinolytic enzymes.

Table 2. Growth and production of pectinolytic enzymes by free and immobilized *Aspergillus niger* EF97 strain.

Enzymatic activity	Free cells	Immobilized cells
Polygalacturonase PG (U/ml)	9.8	18.2
Pectinesterase PE (U/ml)	2.5	0
Pectinlyase PL (U/ml)	1.9	0
Rategrowth(h^{-1})	0.06	0.03

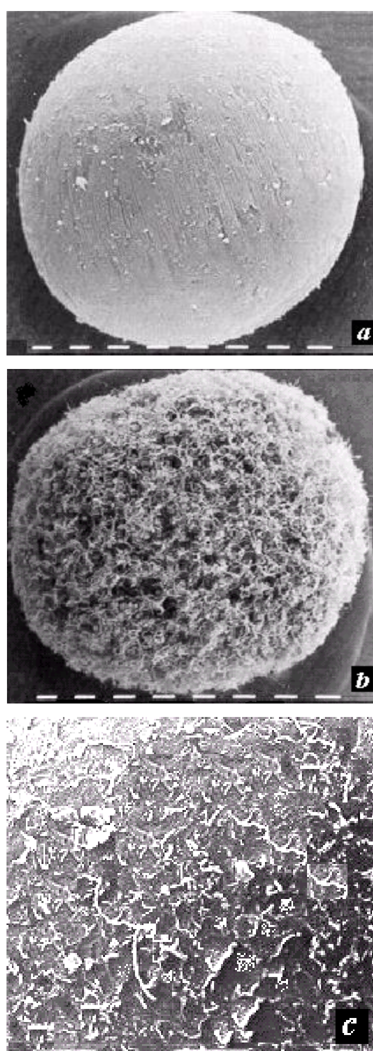

Fig. 3. Electronic photo of a calcium alginate bubble contains the immobilized *Aspergillus niger* concentrate. a) after 1 cycle, b) after 6 cycles. c) detail of the surface of the bead b. (Wild MPS 45 / MPS 51 S.) Cursor = 0.1 mm.

Effect of substrate concentration on biosynthesis

The production of pectinases by *Aspergillus niger* is influenced by temperature, pH, aeration, incubation time, pectin concentration and agitation. Optima were obtained by the experimental planning method (Carlson and Nordahl, 1993). The results of optimizing these conditions was done in another separate work. However, the effect of substrate concentration (pectin) on the polygalacturonase (PG) activity of immobilized cells has been adequately studied and the results are assigned in Fig. 4.

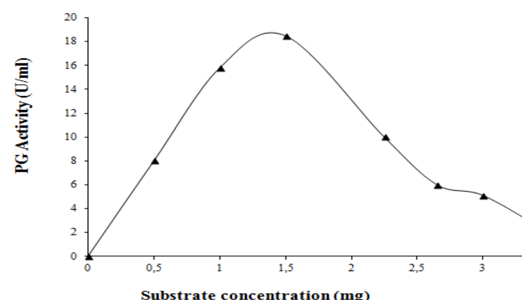

Fig. 4. Effect of substrate concentration on Polygalacturonase (PG) activity.

Fig. 4 shows that the production of polygalacturonase (PG) activity depends on the concentration of pectin in the culture medium. The PG activity of immobilized cells in full growth increased from the 0.1% concentration of pectin to reach the maximum at a concentration of 1.5%, and then decreased for higher concentrations. This result suggests that changes in enzyme biosynthesis (PG) in the presence of a range of concentrations of the pectic powder can be attributed to the combined action of catabolic repression and the effect of the calcium alginate. Our results thus provide evidence for a high level of PG synthesis control by immobilized cells. The sharp rise in activity of the enzyme after the addition of the substrate in the culture medium justifies the activation model of the enzyme polygalacturonase PG. However, above the substrate mass concentration of 1.5%, there is a significant repression of the polygalacturonase PG enzyme in the immobilized cells.

The Fig. 5 shows that after a latency period of 60 min, the addition of 1.5% of substrate to the free *Aspergillus niger* EF97 mycelium induced a rapid

increase in growth, which is expressed in CO2. (g /l) released by free cells and a reduction in PG activity.

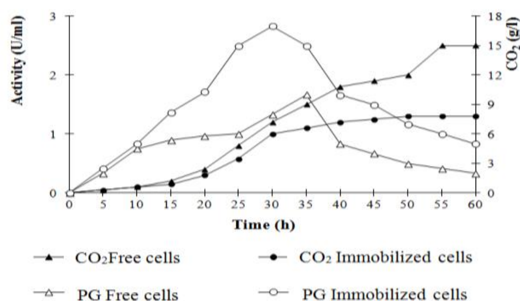

Fig. 5. Effect of immobilization on the growth of the *Aspergillus niger* strain and on the enzymatic activity produced (1.5% of the pectin).

For immobilized cells, there is a 50% reduction in growth and an increase in PG activity. Similarly, the 8-hour lag period of the maximum PG activity between free and immobilized cells may be due to the time required to establish an effective level of induction and to reduce the concentration of some repressors. Say the glucose produced by the pectinolytic activity.

The effects of immobilization mentioned above on the metabolism of the *Aspergillus niger* strain will contribute to the understanding of its new physiological behavior. These effects are caused by the creation of new conditions different from those in free culture. The interpretation of the results of the artificial immobilization allows their exploitation in the agro-food industry. For this reason, we plan the evaluation of metabolic activities within the immobilization matrix (alginate). Thanks to industrial biotechnology, these results would allow the exploration of this large-scale cellular technique in the food industry.

Stability of free and immobilized enzyme activity

Under the conditions described in this work, free and immobilized *Aspergillus niger* EF 97 strain is able to improve the yield of essential oils by 17%. Stability of immobilized free pectinase was examined with batch reactions (repeated). We found that repeated use of free and immobilized pectinases in enzymatic (plant-enzyme) treatments revealed that the capacity of free pectinases fell.

The enzyme was deactivated rapidly after the second cycle. However, Fig. 6 shows that after 6 cycles, the enzyme immobilized on calcium alginate retained 53% of its initial activity. Immobilized pectinases retained some stability and showed long-term efficacy during batch reactions. The stability and productivity of immobilized enzymes during batch reactions has been demonstrated in several studies (El-Aassar *et al.,* 1990; Seung *et al.,* 2001, Elagli, 2015).

This knowledge can solve a big problem of the solid waste of the industrial units by fermentation and we quote as an example, the lignocellulosic waste which is rich in pectin. These wastes supplemented with mineral sources, are of great interest in the production of pectinolytic enzymes by immobilized cells with high specific productivity of the Polygalacturonase enzyme PG.

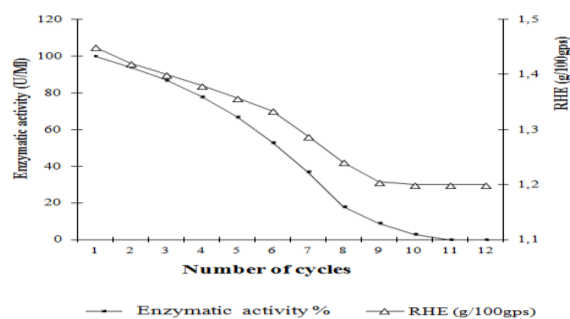

Fig. 6. Distillation of essential oils after enzymatic treatment with recycled immobilized cells.

Conclusion

The improvement in the yield of essential oils depends on the technology of production and exploitation of the hydrolytic enzymes used under controlled thermodynamic conditions. In a more immediate manner, the enzymatic operations have characteristics that now justify, in comparison with chemical catalysts, enzymes operating at low temperatures, their use inevitably results in savings of thermal energy.

The development of knowledge has made it possible to market specific pectolytic preparations possessing: A good ratio: PL, PG and PE, preparations having the immobilization technology making it possible to increase the kinetics of degradation and a good stability of enzymatic activities.

In research perspectives, we will consider developing techniques of confocal scanning microscopy and nuclear magnetic resonance to better control the metabolic pathways involved in the production of the Polygalacturonase enzyme (PG). And to improve the process of bio transformation and reduce the costs of pectinase enzyme production, we will try in a next step, to replace the pectin with lignocellulosic waste.

References

Albersheim P. 1966. Pectin lyase from fungi In: Methods in Enzymology, Vol. (Neufeld EF. and Ginsburg V. EdS.). Academic Press, New York. 8, 628-635.

Barman S, Sit N, Badwaik LS, Deka SC. 2015. Pectinase production by Aspergillus niger using banana (*Musa balbisiana*) peel as substrate and its effect on clarification of banana juice. J Food Sci Technol. 2015 Jun **52(6),** 3579-89.

Bayoumi RA, Yassin HM, Swelim MA, Abdel-All EZ. 2008. Production of bacterial pectinase(s) from agro-industrial wastes under solid state fermentation conditions. J Appl Sci Res **4(12),** 1708–1721.

Carlson R, Nordahl A. 1993. Exploring organic synthetic, eperimental procedures. Topics in current chemistry **166,** 1-64.

Dijksterhuis J, Wösten H. 2013. Development of *Aspergillus niger* Studies in Mycology 74 (March 2013).

El-Aassar SA, El-Badry M, Abdel-Fattah AF. 1990. The biosynthesis of proteases with fibrinolytic activity in immobilized cultures of *Penicillium chrysogenum* H9. Appl Microbiol Biotechnol **33,** 26-30.

Elagli. 2015. Contribution à l'intensification de procédés appliqués à la protéolyse enzymatique Etude microcinétique en réacteur microfluidique et conception assistée par plasma froid d'un microréacteur à enzyme immobilisé" thèse de doctorat, IUTA–Polytech'Lille, France.

Eloutassi N. 2004. Elaboration de procédés biotechnologiques pour la valorisation du romarin (*Rosmarinus officinalis*) marocain. Thèse pour l'obtention de Doctorat ES Sciences en Biotechnologie. Université Sidi Mohamed BenAbedellah, FSDM, Fès, Maroc, 96-97.

Herron SR. and Benen JA. 2000. Structure and function of pectic enzymes: virulence factors of plant pathogens. Proceedings of the National Academy of Sciences of the United States of America **97(16),** 8762-8769.

Hoa BT, Hung PV. 2013. Optimization of nutritional composition and fermentation conditions for cellulase and pectinase production by *Aspergillus oryzae* using response surface methodology. International Food Research Journal **20(6),** 3269-3274.

Ibrahim M, Briandet R, Mistou MY, Chrétien A, Tremblay J, Kulakauskas S. 2004. Immobilisation des lactocoques" Lait **84,** 103–114.

Jamai L, Sendide K, Ettayebi K, Errachidi F, Hamdouni AO, Tahri JMA, McDermott T, Ettayebi M. 2001. Physiological difference during ethanol fermentation between calcium alginate immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. FEMS Microbiol Lett **2,** 375-379.

Kassim C. 2012. Vinification continue avec levures immobilisées: Analyse du système et conception du réacteur industriel. thèse de Doctorat université de Touluse.

Khairnar Y, Krishna KV, Boraste A, Gupta N, Trivedi S, Patil P, Gupta G, Gupta M, Jhadav A, Mujapara A, Joshi B, Mishra D. 2009. Study of pectinase production in submerged fermentation using different strains of *Aspergillus niger*" International Journal of Microbiology Research, ISSN: 0975-5276, **Volume 1, Issue 2,** pp-13-17.

Martos MA, Martinez Vazquez F, Fernando, Benassi A, Roque Hours. 2009. Production of pectinases by *A. niger*: influence of fermentation O. conditions" Braz. arch. biol. technol. **vol. 52 no. 3** Curitiba May/June 2009

Mazni, I. 2007. Essential Oil Extraction from Gaharu". Retrieved July 30,2008, http://eresources.perpun.net.my/repository/ tabstractl.php7id-4238.

Miller GL. 1969. Use of Dinitrosalycylique acide reagent for determination of reducing sugar. Anal. Chem. **31,** 426-428.

Minussi RC, Soares-Ramos JRL, Coelho JC, Olzany SD. 1998. Sugar-cane juice induces pectin lyase and polygalacturonase in *Penicillium griseoroseum*. Revista de Microbiologia. Rev. Microbiol **29,** 4.

Nazneen A, Morshed MA, Uddin A, Begum F, Sultan T, Azad AK. 2011. Production of Pectinase by *Aspergillus niger* Cultured in Solid State Media" International Journal of Biosciences **Vol. 1, No. 1,** p. 33-42, 2011.

Ouis N. 2015. Etude chimique et biologique des huiles essentielles de coriandre, de fenouil et depersil" thèse de doctorat université d'Oran 1faculté des sciences exactes et appliquées.

Pinheiro VE, Desagiacomo CCV, Michelin M, Maller A, Monteiro LMO, Jorge JA, Polizeli MDLTD. 2017. Neosartorya glabra polygalacturonase produced from fruit peels as inducers has the potential for application in passion fruit and apple juices. Brazilian Journal of Food Technology **20,** pp 1-11.

Pochon JP, Tordux. 1962. Techniques d'analyse en microbiologie du sol. Collection Technique de base France 101.

Rigal D. 2001. Recherches sur l'inhibition du brunissement enzymatique : utilisation de préparations enzymatiques, substituts aux sulfites. Thèse pour obtenir le grade de Docteur de l'université de droit, d'économie et des sciences d'Aix-Marseille III, France.

Robert A, Ellen S, Connie AN, Oorschot V. 1981. Introduction to food borne fungi. Central bureau Voor Schimmel cultures, Institute of royal Netherlands, Academy of arts and sciences.

Rogerson FSS, Vale E, Grande HJ, Silva MCM. 2000. Alternative processing of port-wine using pectolytic enzymes. Cienc. Tecnol. Aliment **2(5),** 222-227.

Santé Canada. 2017. Avis de modification à la Liste des enzymes alimentaires autorisées visant à permettre l'utilisation de la mannanase d'Aspergillus nigerAE-HCM dans le concentré de café liquide. Numéro de référence: NOM/ADM-0103

Selmaoui K, Ouazzani Touhami A, Mouria A, Benkirane R, Douira A. 2017. Détection de l'activité enzymatique pectinolytique et cellulolytique des champignons responsables de la pourriture des pommes en conservation" International Journal of Innovation and Scientific Research **Vol. 30 No. 2** pp. 242-250.

Seung L, Ki-Young L, Yong-Bok L, Ki-Bang S. 2001. Immobilization of levan fructotransferase for the production di-fructose anhydride from levan. Biotechnology Letters **23,** 1335-339.

Silva S, Ramon Portugal F, Silva P, Abreu S, Teixeira Da Silva M, Strehaiano P. 2003. Malic acid consumption by dry immobilized cells of *Schizosaccharomyces pombe* "American Journal of Enology and Viticulture" **54,** 50-55.

Tan LU, Chan M, SaddIen JN. 1984. A modification of lowry method for detecting protein in media containing lignocellulosic substrates. Biotechnol. Lett **3,** 199-204.

Vries PV, Visser J. 2001. *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiology and Molecular Biology Reviews **65(4),** 497-522.