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PHYSIOLOGICAL REGULATION OF PROTEASE AND ANTIBIOTICS IN *PENICILLIUM* SP. USING SUBMERGED AND SOLID STATE FERMENTATION TECHNIQUES

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Abstract

A fungal strain belonging to the genus *Penicillium* was isolated from soil sample and has been diagnosed as *Penicillium* sp. according to its morphological characteristics of the colonies on solid media and also microscopical examination of the fungal parts. Antibiotics, protease activity and pH values were determined after cultivation of the fungus using submerged fermentation (SF) and solid state fermentation (SSF). The two different patterns of fermentation processes seem to influence the physiological behavior of the fungus differently. Experiments were made using nutrient broth medium (N.B) for SF and wheat bran in SSF. The pH values were adjacent to 5.5. Wheat bran was enriched with fish scales and egg shale in a ratio of (1:2:0.005 w/w) and the mixture was moistened by adding (30 ml) whey solution. After 7 days of incubation, the pH value of SF was increased to 8.0 at 30ºC. The SF was appeared efficient for antibiotics production. Using well diffusion technique the extracted antibiotics solution was active against some pathogenic bacteria such as *Staphylococcus aureus, E. coli, Proteus* sp., *Salmonella* sp., *Pseudomonas aeruginosa* and *Streptococcus* sp. In SSF relative proteases concentrations were found to be highly reactive than SF. This was proved by the appearance of the zone (20 mm and 32 mm) due to the hydrolysis of milk and blood proteins respectively using pH 5.5 at 30ºC for 24 hrs. The activity of proteases was (10.4 U/ml).

Keywords: *Penicillium* species, Physiological behavior, Enzyme production, Antibiotics production, Solid state fermentation.

1. Introduction

Since 1940`s, western industries started to focus on the production of fungal enzymes and specific secondary metabolites such as antibiotics by cultivation of the fungi in liquid media, also called submerged fermentation (SF). Fungi have been known as enzyme producers and some species are being exploited industrially to make amylases, proteases and cellulases. Among others, some species of the genus *Penicillium* have been reported to possess enzymes with high levels of activity [1]. In submerged culture the morphology of filamentous microorganisms varies between two extreme forms, pellets and free filaments, depending on culture conditions and the genotype of the applied strain. A close link between mycelial morphology and productivity has early been identified in important industrial processes such as the citric acid fermentation and several antibiotic fermentations [2].

Protein and other metabolites production by solid substrate fermentation (SSF) using renewable sources received worldwide attention. In developed countries this is an important way of dietary changes for the partial substitution of animal protein with plant protein including many enzymes. Oliveira et al [3] showed that the fungal growth in SSF has advantages over submerged cultures, because this is a natural environment for filamentous fungi. The genus *Penicillium* encompasses a large number of species that are capable of growing on a diverse range of substrates [4].

This study focused on the factors influencing the fungal morphology and metabolites production such as protease enzyme and antibiotics from local isolation of *Penicillium* sp. in both submerged and solid state techniques.

2. Experimental

2.1. Organism and culture conditions

Penicillium sp. strain was isolated from soil sample in Sulaimani City, North of Iraq. The soil sample was dried in an oven at 50°C for overnight, and then serial dilutions were made from the sample, after that 0.1 ml from suitable dilutions (10^{-3}, 10^{-4}, and 10^{-5}) were spread on Potato Dextrose Agar (PDA) and Sabouraud's Agar (SA) plates. All plates were incubated at 30°C for 3-5 days. All fungal colonies, which were appeared on the plates at 30°C, were selected and their morphological features were registered, then the microscopical examination of the fungal parts was done to detect the genus of fungi according to [5].

2.2. Inoculum preparation

The spore cells were counted in the inoculum size according to [6]. The spores were harvested from culture plate grown for 5-7 days in Petri dishes containing PDA at 30°C by adding sterile water to stock culture. A stock suspension was prepared and adjusted to 2×10^{8} spore/ml using direct counting by light microscope.

2.3. Production of protease and antibiotics

2.3.1. Submerged fermentation

Nutrient broth (100 ml) with a pH 5.5 in a flask was used. The flask was inoculated with 2 ml from the spore suspension, in which it contains 2×10^8 spore/ml, and then the flask was incubated at 30°C for 7 days. The broth culture was centrifuged in a centrifuge (NUVE NF 615, NUVE, Turkey) at 5000 rpm for 15 min. Then the protease activity and the antibiotics production were detected in the supernatant as mentioned in articles (2.5) and (2.6) respectively of this experimental.

2.3.2. Solid state fermentation

Erlenmeyer flasks (250 ml) contained wheat bran, fish scales and egg shale in a ratio (1:2:0.005) wt/wt were used. The mixtures in the flasks were moistening with whey solution (30 ml/flask). The media were adjusted to a pH 5.5 before sterilization. After inoculation with 2×10^8 spore/ml of the isolate, the flasks were incubated at 30°C for 7 days [7]. The protease enzyme and antibiotics solution were extracted from the fermentation medium by adding 30 ml distilled water to the fermentation flasks with stirring using magnetic stirrer for about 20 min. The mixture was centrifuged at 5000 rpm for 25 min to remove the fungal parts and the residues medium. The supernatant (crude solution) was clarified by filtration through glass wool and the filtrate was stored in a freezer (-20°C) for further works.

2.4. Total protein assay

Protein concentration was determined by the Biuret method [8], according to bovine serum albumin as a standard protein.

2.5. Determination of proteolytic activity

Initially, protease activity was tested using skim milk agar and blood agar. For this, 0.01 ml (using graduated micropipette) of crude enzyme produced from local isolate of *Penicillium* sp. was added inside wells in the center of the plates very carefully not to overflow the wells; the plates were then incubated at 30°C for 24 hrs. Proteolytic activity was detected by observing in the presence of clear zones [9]. After initial screening for proteolytic activity, *Penicillium* sp. strain exhibited activity of proteases and was used throughout the experiment.

Determination of the enzyme activity was performed using the amino acids measurement [10]. The reaction mixture contained 1.9 ml of 1% hemoglobin (previously prepared in acetate buffer 0.2 M, pH 6.0), and 0.1 ml sample (crude enzyme). The reaction mixture was incubated at 37°C for 10 min and then stopped by adding 2 ml of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 3000 rpm for 30 min. The amount of free amino acids groups was measured in the supernatant by reading the absorbency at 280 nm using spectrophotometer (Techomp UV 7500, Techomp, China). Proteases activity is expressed as μmol of free amino acids equivalent per min/ml of the culture filtrate. The same mixture except hemoglobin was used as a control [10].

2.6. Detection of antibiotics production

The same protocol of Chantawannaku *et al.* [9] was followed in an investigation of antibiotics production assay. The only difference is that the protocol was carried out against different bacterial strains (*Staphylococcus aureus, E. coli, Proteus* sp.*, Salmonella* sp.*, Pseudomonas aeruginosa* and *Streptococcus* sp.) previously seeded on nutrient agar plates. According to Cooper [11], activity of biological substances (i.e., antibiotics and enzymes) can be expressed in terms of square of the diameter of the clear zone.

3. Results and Discussion

3.1. Detection of antibiotics production

The results obtained in this study revealed the ability of the local isolate of *Penicillium* sp. to produce extracellular protease and in the same time to produce antimicrobial substances. The isolate was obtained and further identified using the morphological characters of the growth on different culture media. The *Penicillium* sp. is of particular interest due to its production of desired substances such as proteases and antibiotics. The morphological feature of the *Penicillium* growth was green in color on PDA and SA, and it appeared white with little pink color on NA with yellow diffusible pigment (Fig. 1). Diffusible pigments are formed by some species and it was used for fungal identification [5]. Colonies were appeared velvety or woolly on different culture media.

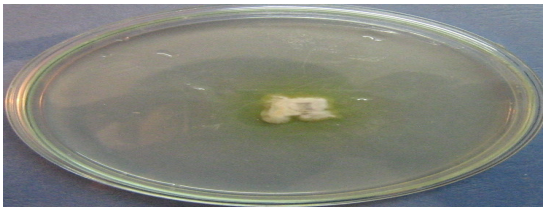

Fig. 1. Macroscopic View of a Growth of the Local Isolate of *Penicillium* sp. on NA Plate at 30°C/72 hrs.

Penicillium is a large genus, with at least 150 species, many with similar morphology. There is also a great deal of variability within many species, and at least 1,000 recognizably different phenotypes eventually may be catalogued. Because of the inherent variability in the genus, only 70 to 80% of isolates, even from common sources, are readily identifiable. Many of the taxonomic keys to identify *Penicillium* sp. are based primarily on morphological criteria. Most of them base identification upon micromorphology, macromorphology, and colors produced in the mycelium or diffused into the growth medium [12]. *Penicillium* species form a well-developed septate mycelium with exhibit distinctive colors such as yellow, orange, red or purple. The tip of unbranched conidiophores may terminate in a cluster of phialides or in a cluster of metulae. In branched conidiophores, each branch terminates in one or more metulae [5]. The conidia are spherical to ellipsoidal, aseptate, and they may be blue-green, grey-green or yellow, according to species (Fig. 2).

Fig. 2. Microscopic View shows the Conidia and the Brush Arrangement of Phialospores of the Local Isolate of *Penicillium* sp. (40x magnification).

3.2. Production of protease and antibiotics

Penicillium species are widespread in nature and are characteristically saprotrophic and many species form toxins and others can cause spoilage of various types of materials. Some are used in manufacturing processes and some produce useful substances such as antibiotics and enzymes. The local isolate of *Penicillium* sp. was revealed high proteases activity on milk and blood agar plates (Figs. 3 and 4). This is justified by the formation of the clear zone of (20 mm) diameter which is resulted from the hydrolysis of milk and blood proteins.

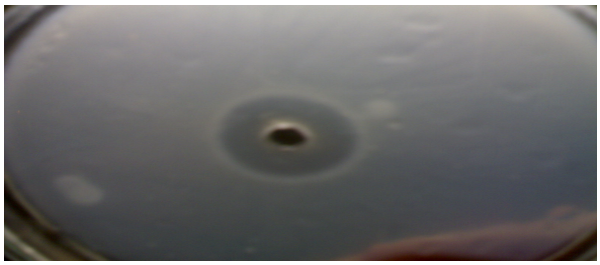

Fig. 3. Activity of Protease Enzyme Produced from Local Isolate of *Penicillium* sp. on Milk Agar Plate at 30°C/24 hrs.

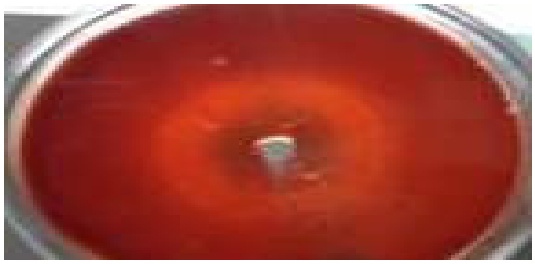

Fig. 4. Activity of Protease Enzyme Produced from Local Isolate of *Penicillium* sp. on Blood Agar Plate at 30°C/24 hrs.

Many antibiotics are produced by microorganisms, predominantly by actinomycetes and by filamentous fungi. Penicillins e.g. produced by *Penicillium chrysogenum* and other species may produce useful antibiotics such as griseofulvin, mycophenolic acid [13]. Table 1 shows the activity of antibacterial agents which were produced from the local isolate of *Penicillium* sp. From the results below and with the same conditions (incubation temperature and the time), the agents affected the growth of both Gram-positive and Gram-negative bacterial species.

Table 1. The Activity of Antibiotics Produced from Local Isolate of *Penicillium* sp. against Different Pathogenic Bacteria.

Test organism	Inoculum size (cell/ml × 10^8)	Inhibition zone (mm)
Staphylococcus aureus	6	19
E. coli	8	17
Proteus sp.	22	10
Salmonella sp.	53	16
Pseudomonas aeruginosa	13	9
Streptococcus sp.	33	14

Investigations show that the producing amount of protease by many microbial cultures varies greatly with the media used and regulatory effects exerted by the carbon sources [14].The results obtained in this work revealed the ability of *Penicillium* sp. to produce extracellular proteases. Figure 5 shows the behavior of enzyme activity, the antibiotics production (inhibition zone) and pH values in case of SF, while Fig. 6 shows the behavior of enzyme activity, antibiotics production (the inhibition zone) and pH values in case of SSF. Growth of the mycelia increased rapidly in the early stages and the color of growth was white and an increase on biomass and antibiotics production occurred when SF was used and the pH values were increased at the end of cultivation from acidic condition to the basic condition and this was probably due to metabolites accumulation resulting from basic compounds that formed during the fermentation.

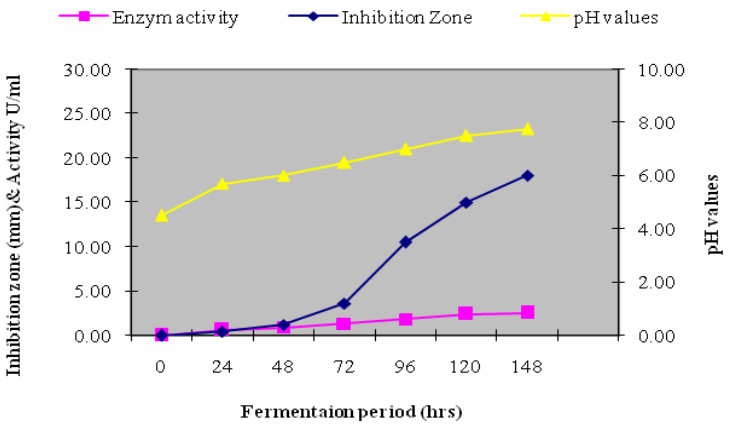

Fig. 5. Antibiotics and Protease Enzyme Production with pH Values in SF by *Penicillium* sp.

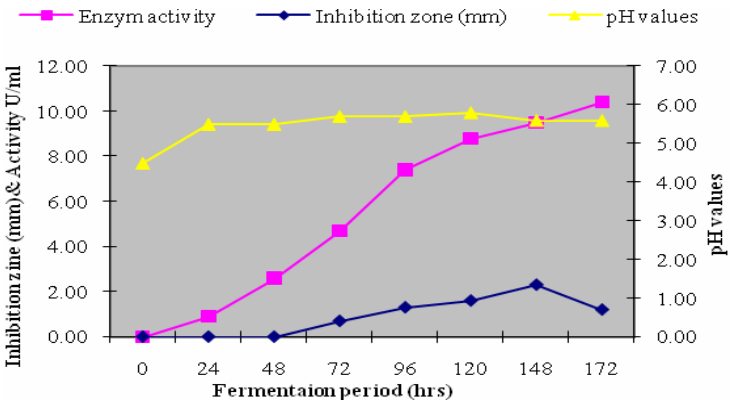

Fig. 6. Antibiotics and Protease Enzyme Production with pH Values in SSF by *Penicillium* sp.

In the case of SSF, the results were completely different, since the enzyme activity was high (10.4 U/ml). The activity of protease here was after 172 hrs of fermentation period while in another study it was occurred after 160 hrs of fermentation period [15]. This is because the germination of fungal spores to young mycelium requires a relatively long time and hence the protease production is delayed. On the other hand, fungi prefer SSF for enzyme production, their hypha capable to penetrate the solid particles of the medium, and hence they need to utilize the residues nutrients in the bottom. From Fig. 6 the antibiotic production was low, and the pH values remain approximately constant.

The growth was green in color and the biomass production was few. This may be due to the fungal reaction about nutrients sources since the medium was rich in carbohydrates [7]. The combination between its nutrients gave to the fungal cells supplement agents for enhancing the growth and for production of the protease enzyme. It contained wheat bran, fish scales and whey protein and these materials were complex so the fungal need to produce enzymes to digest these polymers to simple unites, i.e., amino acids and polypeptides for its growth and reproduction. However, for higher substrate concentrations, the growth is typically semi-associated since product formation takes place during the growth and stationary phases. Various microorganisms and cultivation media have been studied for protease production [4, 16-18].

4. Conclusions

The results presented here suggest the importance of local isolate of *Penicillium* sp. because this fungus is very efficient to produce the extracellular protease and antibiotics production in both SSF and SF respectively. Also, this study indicates the importance of solid state fermentation technique, since it is very simple in application, obtaining high yield products, less energy and aeration requirements in comparison with liquid state fermentation. This strategy might be considered for large scale applications for production of industrial enzymes. The different data obtained

in the study can be adopted as a starting point for an experimental optimization of *Penicillium* sp. growth for large–scale protease production in SSF and antibiotics production in SF.

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