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FACTORS AFFECTING THE (PRODUCTION OF EXTRACELLULAR NEUTRAL PROTEASE) BY *STREPTOMYCES MICROFLAVUS*

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ABSTRACT

In order to produce neutral protease from *Streptomyces microflavus*, it was cultivated in basal medium containing soluble starch, potassium nitrate, dipotassium hydrogen phosphate, magnesium sulphate, sodium chloride, calcium carbonate and ferrous sulphate. Protease production increased with decreasing the ratio of culture broth to vessel volume 1:5 and shaking at 150 rpm. Protease production was low when pH was < 5 or > 9. The productivity of protease decreased sharply when the incubation temperature increased from 30 to 45^{0}C. The maximum yield of protease was obtained at the third day of incubation. Soluble starch and yeast extract were the best carbon and nitrogen sources, respectively. Supplementation with calcium carbonate enhanced protease production. In addition, the dipotassium hydrogen phosphate was the best phosphorous source. Para chloromercuribenzoic acid and phenyl methylsulfonylfloride had significant inhibitory effect on protease production.

INTRODUCTION

Streptomycetes are Gram-positive, mycelium-forming soil bacteria that include many species and are considered among the most important producers of antibiotics and enzymes (**Edwards, 1993**). They are able to degrade many macromolecules such as lipids, starch, chitin, xylan, pectin and proteins. Proteolytic cleavage of peptide bounds is one of the most frequent modifications of proteins. The responsible enzyme is recognized as serine proteases, cysteine proteases, aspartic proteases and metalloproteases (**Barrett 1986**). Proteases are common among tissues, plants, fungi and bacteria. Streptomycetes proteases have been purified and characterised from various sources (Henderson *et al* 1987; **Bascaran *et al* 1990; Lampel *et al* 1992; Aphale and Strohl 1993**). Many workers emphasized that some species of *Streptomyces* can produce protease (**Kang *et al* 1995; Jain *et al* 2003 and Uyeda, 2004**).

Proteases represent one of the three largest groups of industrial enzymes, nearly accounting 60% of total worldwide enzyme sales. Proteases refer to a group of enzymes whose function are to break down proteins to their basic building blocks of amino acids and also referred as proteolytic enzymes or proteinases. Proteases are essential for physiological processes such as cell growth and death, blood clotting, tumour growth and bone remodelling. In addition, proteases have many industrial and agricultural applications such as processing of leather and wool as in alkaline, acidic and neutral proteases, food and beverage production as acidic protease, cheese and baker manufacture as neutral protease, the detergent industry, sewage treatment and silver recovery as alkaline protease, and finally as biological control of certain diseases.(**Vinci, 1993**).

The objective of this study is to investigate the factors affecting the production of extracellular neutral protease by *Streptomyces microflavus*.

MATERIALS AND METHODS

Microorganism

Twenty three streptomycetes isolates from Egyptian agricultural soil were identified and showed proteolytic activity. All the isolates were examined quantitatively for protease production.

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Streptomyces microflavus isolated from the soil of Plant Island, Aswan, Egypt showed highest neutral protease production.

Culture medium and growth conditions

Streptomyces microflavus was cultivated at 28°C on a slant of starch-nitrate agar medium. Spores were harvested with sterile water which was adjusted to give 10^7 to 10^8 spores per ml. Submerged culture medium contained (g/L) soluble starch 20.0, KNO_3 2.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, NaCl 0.5, $CaCO_3$ 3.0 and $FeSO_4.7H_2O$ 0.001 at pH 7.0-7.2 was used. The broth was inoculated with 1.0×10^6 spores per ml and shaken at 28°C and 150 rotation/min for 3 days.

Protease assay

Protease activity in culture broth was determined using a modification of Anson's method (**Yang and Huang, 1994**). The absorbance of liberated tyrosine in the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that produced an absorbance at 280 nm equivalent to 1µmol of tyrosine in one minute under the assay conditions.

Parameters controlling protease production

- Flasks containing 25, 50, 75 and 100 ml of basal medium were cultivated.
- The culture was inoculated and shaked at 50, 100, 150 and 200 rpm.
- The initial pH of the basal medium was adjusted with 0.1 mol NaOH or 0.1 mol HCl from pH 4.0 to 10.0.
- The culture was cultivated for 0, 2, 3, 4, 5, 6 and 7 days, respectively.
- The culture was incubated at 20, 25, 28, 30, 35, 40, 45 and 50 °C.
- The carbon source in the basal medium was soluble starch which replaced by 2% (w/v) of D-glucose, D-fructose, galactose, L-arabinose, lactose, D- mannitol, sucrose and glycerol.
- The optional concentration of potassium nitrate, used as a nitrogen source, in the basal medium was changed in equimolar. KNO_3 was replaced by ammonium nitrate, sodium nitrate, ammonium sulphate, yeast extract, peptone, casein, cystine and aspartic acid.
- The concentration of the inorganic salt (calcium carbonate) in the basal medium ranged from 0.0 to 2.5 g/L.
- The phosphate source in the medium was di-potassium hydrogen phosphate. It was replaced in equimolar by potassium dihydrogen phosphate, ammonium phosphate and sodium phosphate.
- Different mineral ions such as $AgNO_3$, $CaCl_2$, $MnCl_2$, $ZnCl_2$ and $FeSO_4$ in the concentration of 0.0065 mmol were used.
- Different inhibitors were used such as Tween 80, Urea, EDTA (Ethylene diamine tetraacetic acid), P.CMBA (Para chloromercuribenzoic acid) and PMFS (Phenyl methylsulfonylfloride).

RESULTS

Protease production

Streptomyces microflavus isolates were cultivated in different kinds of protease-producing media. The selected medium previously mentioned in materials and methods showing the highest protease activity among the other different tested media (each ml of broth medium contained 0.528 units), was chosen as the best medium in this study for protease production.

Parameters controlling neutral protease production

1- Volume ratio

Protease production was affected by changes in volume ratio (**Fig. 1**). The high productivity was attained to a volume ratio of 1:2.5 and 1:5.0 yielded 0.449 and 0.528 units of protease, respectively. Protease production is significantly decreased by increasing volume.

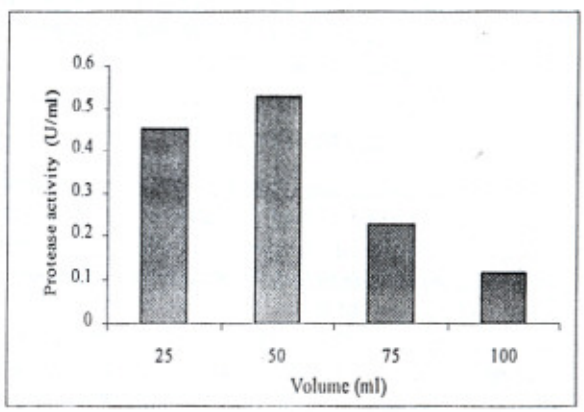

Fig. 1. Effect of volume ratio on neutral protease production from *Streptomyces microflavus*

2- Shaking

An increase in protease activity was detected from static culture (0.097 U/ml) till 100 rpm (0.245 U/ml) as shown in **Fig. (2)**. Abrupt increase in protease activity was observed at 150 and 200 rpm (0.528 and 0.506 U/ml, respectively).

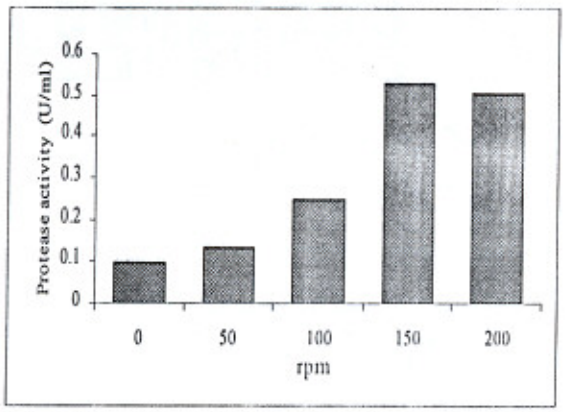

Fig. 2. Effect of shaking on neutral protease production from *Streptomyces microflavus*

3- Initial pH

Protease activity was undetectable below pH 5 and above pH 9 **(Fig. 3)**. Drastic increase in activity was detected between pH 5 (0.017 U/ml) and pH 6 (0.371 U/ml). Protease reached the maximal yield at initial pH 7.0 (0.528 U/ml). In addition, sudden decrease in protease activity appeared at pH 8 and 9 (0.098 and 0.004 U/ml, respectively).

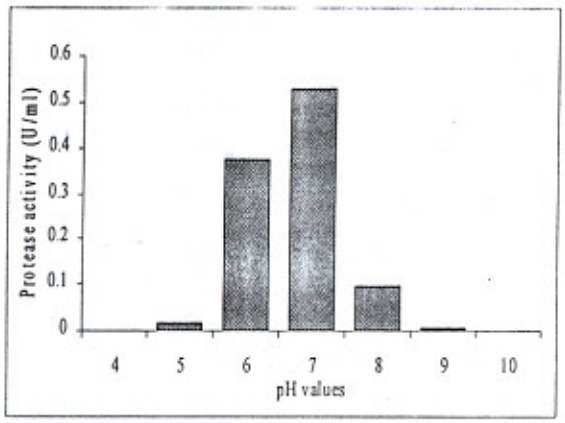

Fig. 3. Effect of pH on neutral protease production from *Streptomyces microflavus*

4- Incubation temperature

Temperature was an important factor for protease activity. Each ml of broth could support 0.513-0.528 units of protease at temperature 28-30^{0}C **(Fig. 4)**. The value decreased to 0.1 U/ml of protease at 45 ^{0}C. Incubation temperature at 50^{0}C showed completely inhibition on protease production.

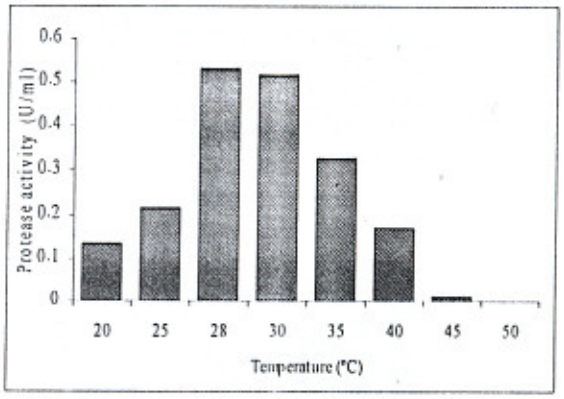

Fig. 4. Effect of temperature on neutral protease production from *Streptomyces microflavus*

5- Incubation period

Protease activity was detected only at the 2nd day of incubation period **(Fig. 5)** in a very weak activity (0.066 U/ml). The maximum yield was obtained at the 3rd day of incubation (0.528 U/ml) followed by gradually decreasing in activity till the 6th day (0.025 U/ml). No activity was detected at the 7th day of incubation.

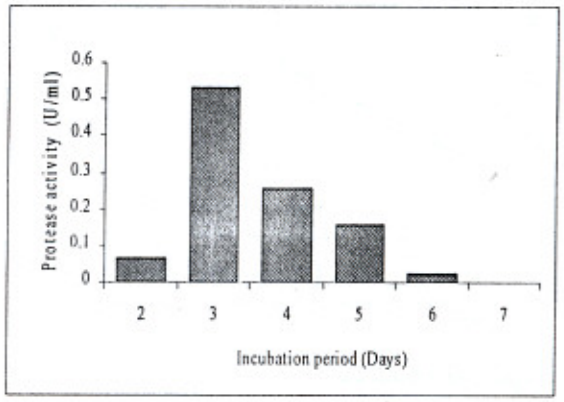

Fig. 5. Effect of incubation period on neutral protease production from *Streptomyces microflavus*

6- Carbon sources

The effect of different carbon sources on the production of protease is shown in **Fig. (6)**. Soluble starch was the most effective carbon source for protease production (0.528 U/ml) followed by D-fructose, galactose, L-arabinose, lactose and D-mannitol. Sucrose, glucose and glycerol inhibited protease production.

Moreover, the effect of different concentrations of starch on the production of protease is illustrated in **Fig. (7)**. Completely inhibition of protease production was obtained with 5 g/L of starch. Increasing of protease production was detected till 20 g/L (0.528 U/ml) followed by gradual decreasing till 30 g/L (0.30 U/ml).

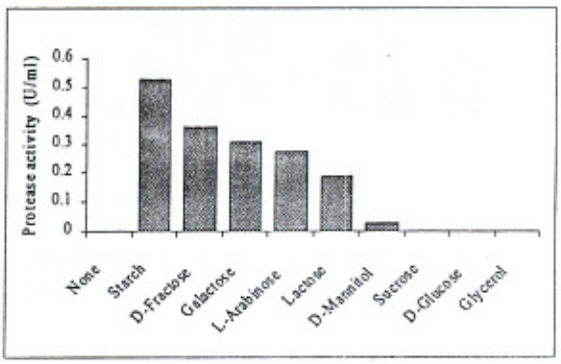

Fig. 6. Effect of different carbon sources on neutral protease production from *Streptomyces microflavus*

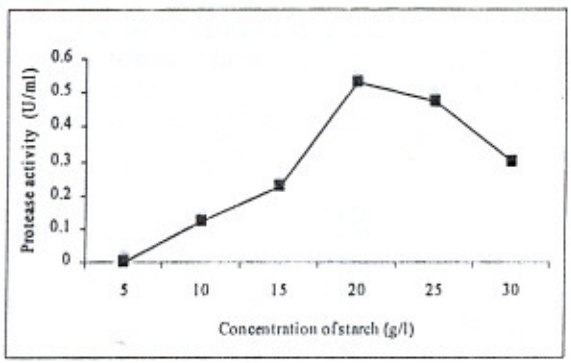

Fig. 7. Effect of starch on neutral protease production from *Streptomyces microflavus*

7- Nitrogen sources

The effect of nitrogen sources on protease production is presented in **Fig. (8)**. Yeast extract was the best nitrogen source for protease activity (0.662 U/ml) followed by potassium nitrate (0.528 U/ml). The other tested nitrogen sources showed significant differences. Aspartic acid showed the minimum protease activity (0.180 U/ml).

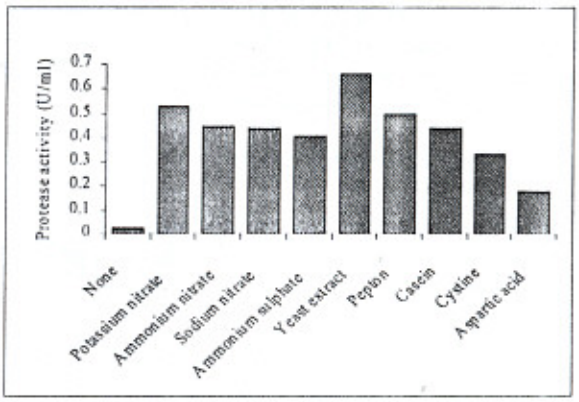

Fig. 8. Effect of different nitrogen sources on neutral protease production from *Streptomyces microflavus*

In addition, different concentrations of yeast extract were used to check their effect on protease activity **(Fig. 9)**. By increasing the concentration of yeast extract, the maximum activity of protease was noticed at 3.8 g/l (0.726 U/ml). Then protease activity decreased by increasing the concentration of yeast extract.

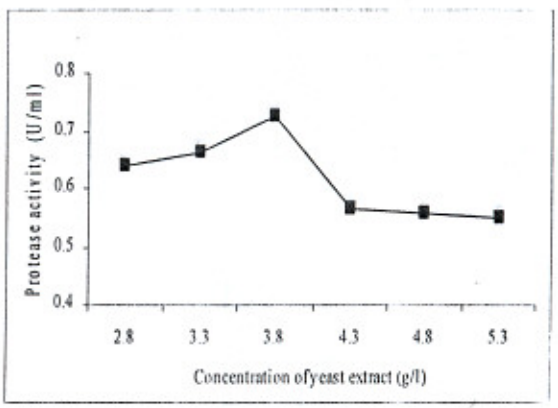

Fig. 9. Effect of yeast extract on neutral protease production from *Streptomyces microflavus*

8- Calcium carbonate ($CaCO_3$)

The effect of different concentrations of $CaCO_3$ on protease production is shown in **Fig. (10)**. Relative high production was shown between 2 and 3.5 g/L (0.623 and 0.645 U/ml). The maximum yield was detected at 3 g/l (0.726 U/ml).

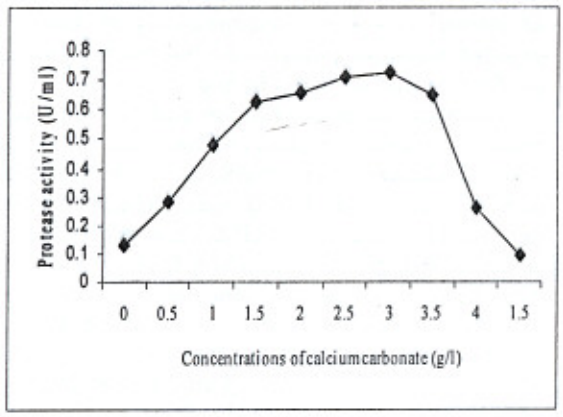

Fig. 10. Effect of calcium carbonate on neutral protease production from *Streptomyces microflavus*

9- Phosphate sources

Figure (11) showed the effect of different sources of phosphate on protease production. Maximum proteolytic activity secretion occurs with K_2HPO_4 (0.726 U/ml) followed by KH_2PO_4 and $NH_4H_2PO_4$. The minimum protease activity was detected with sodium phosphate (0.252 U/ml).

Moreover, different concentrations of K_2HPO_4 were tested for neutral protease production **(Fig. 12)**; maximum yield was obtained with 1.0 g (0.726 U/ml). The protease production decreased with increasing concentrations of dipotassium hydrogen phosphate from 1.5 g/L (0.515 U/ml) till 2.5 g/L which showed completely inhibition of protease activity.

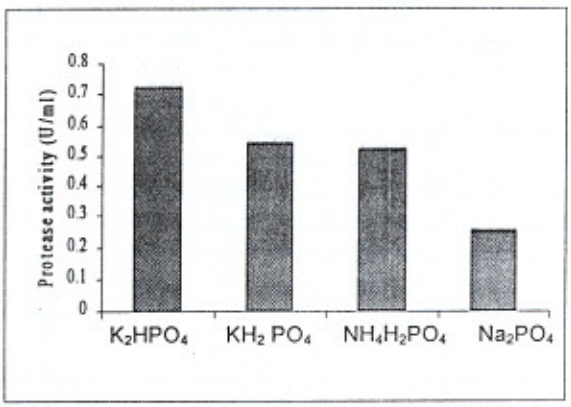

Fig. 11. Effect of different sources of phosphate on neutral protease production from *Streptomyces microflavus*

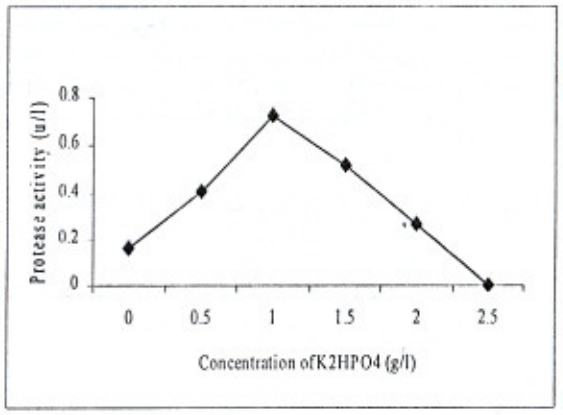

Fig. 12. Effect of di-potassium hydrogen phosphate on neutral protease production from *Streptomyces microflavus*

10- Mineral ions

The effect of mineral ions on neutral protease production is shown in **Fig. (13)**. Ag^+ ion inhibit protease production. The proteolytic production reaching the maximum activity with supplementation of Fe^{2+} (0.726 U/ml) and sharply decreased with Ca^{2+} (0.363 U/ml), Mn^{2+} (0.173 U/ml) and Zn^{2+} (0.077 U/ml), respectively.

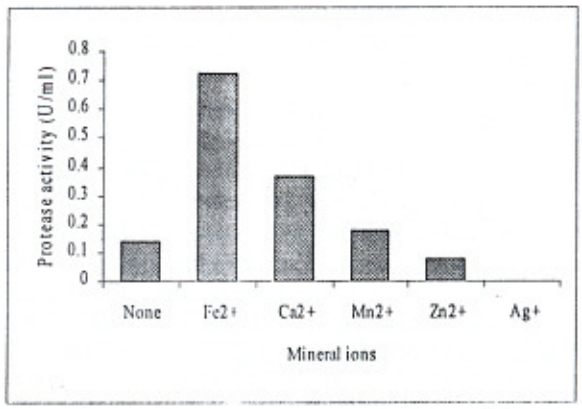

Fig. 13. Effect of different mineral ions on neutral protease production from *Streptomyces microflavus*

11- Inhibitors

Effect of different inhibitors on protease production is illustrated in **Table (1)**. Concentration of 0.1 mmol of P.CMPA and PMFS inhibit protease activity. Maximum residual activity was indicated with Tween 80 at 0.08 and 0.1 mmol concentration (49

and 45%). About two third of the activity was inhibited by urea from 0.2 till 0.8 mmol concentrations (39, 36, 33 and 32%). Residual activity in case of EDTA ranged from 24 to 7% at 0.08 and 0.6 mmol, respectively. Complete inhibition was observed over 0.8 mmol.

Table 1. Effect of different inhibitors on neutral protease production from *Streptomyces microflavus*

Inhibitors	Residual activity (%)				
Conc. Mole	Tween80	Urea	EDTA	P.CMBA	PMFS
0.08	49	42	24	10	5
0.1	45	40	18	0	0
0.2	38	39	17	0	0
0.4	21	36	14	0	0
0.6	5	33	7	0	0
0.8	0	32	0	0	0
1.0	0	0	0	0	0

DISCUSSION

Research efforts have been directed mainly toward physical and nutritional parameters. In addition, no defined medium has been established for the best production of proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

The production of proteases is dependant on the availability of both carbon and nitrogen sources in the medium. The present study indicates that soluble starch was the best carbon source for neutral protease production followed by fructose and galactose. Increasing of alkaline proteases was recorded by several workers using different sugars such as fructose (**Sen and Satyanarayana, 1993**) and lactose (**Malathi and Chakrabory 1991; De Azeredo *et al* 2003**). On the other hand, glucose, sucrose and glycerol showed inhibitory effects on neutral protease production from *Streptomyces microflavus*. Although some carbon and nitrogen sources showed the stimulating effect on microbial growth, they had an inhibitory effect on the biosynthesis of primary metabolites because of organic acid accumulation, oxygen depletion and glucose catabolic repression. The same phenomena was also found in protease production by *Streptomyces rimosus* (**Yang and Lee 2001; Seong *et al* 2004**). The optimum production of neutral protease from *Streptomyces microflavus* was detected with 20 g/l of starch. The high concentrations of starch repressed protease production. The neutral protease production was inhibited by 5 g/l of starch.

Although complex nitrogen sources are usually used for protease production, the requirement for a specific nitrogen supplement, differs from microorganism to the other. Using yeast extract as a nitrogen source, which provided several micronutrients, vitamins and growth promoting factors, stimulated the production of neutral protease in *Streptomyces microflavus*. Several researchers also reported that yeast extract promoted the biosynthesis of protease in *Streptomyces* sp. (**Laluce and Molinari, 1977; Yang and Lee, 2001**). Supplementation of peptone and casein stimulate the microbial growth and they were hydrolysed to small molecules by protease. The use of inorganic nitrogen sources supported a moderate production of protease.

For many years, shaker flasks have been employed as the usual tool for laboratory studies with aerobic microbial processes. There is an increase correlation between oxygen absorption rate and flask liquid volume. In the present work, no direct oxygen measurement was made, but the relationship between the volume of medium and enzyme secretion suggests the necessity of an adequate oxygen supply for the biosynthesis of the enzyme by *Streptomyces microflavus*.

Volume ratio of 1:5 (v/v) and shaking at 150 rpm were optimal for neutral protease production from *Streptomyces microflavus*. High volume ratio (1:10) did not favour neutral protease production, while low volume ratio (1:2.5) postponed the biosynthesis of protease. During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. **Yegneswaran and Gray (1991)** reported that high concentrations of oxygen have a stimulating effect on microbial growth and enzyme production. This indicated that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

Streptomyces microflavus had a high metabolic activity when the initial pH was controlled at 7.0. The addition of calcium carbonate in the medium increased protease production due to the regulation of pH for microbial growth and metabolic biosynthesis (**Vinci *et al* 1993**). K_2HPO_4 regulate substrate pH and stimulate protease production. The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane (**Moon and Parulekar, 1991**). Temperature is another critical parameter that has to be controlled and varied from organism

to the other. The mechanism of temperature control of enzyme production is not well understood (**Chaloupka, 1985**). However, studies by **Frakena *et al* (1986)** showed that a link was existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake. The optimal temperature for neutral protease production in *Streptomyces microflavus* ranged between 28-30^{0}C. Completely inhibition in protease production detected at 50^{0}C. The obtained results are in agreement with previous reports for protease production from streptomycetes (**Kim and Lee 1996**).

The obtained results indicate that dipotassium hydrogen phosphate gave the maximum neutral protease production. Potassium phosphate has been used as a source of phosphate in most studies (**Moon and Parulekar 1991; Mao *et al* 1992**). The best results were obtained on a medium with disubistituted potassium phosphate when use at 0.1% (w/v); increasing of this concentration showed a repression in protease production. The same result was reported by **Moon and Parulekar (1991)**. It is known that the absence of mineral sources of phosphorus in the medium causes a substantial drop in the activity and a decrease in the intensity of growth of the culture, which is due not only to the significance of phosphorous as an element of nutrition, but also to the buffering of solutions of its salts.

Divalent metal ions such as iron and calcium showed maximum neutral protease production followed by manganese. Ag+ completely inhibited the production of neutral protease. The requirement for specific metal ions depends on the source of enzyme (**Kumar and Takagi, 1999**).

The results indicated completely inhibition of neutral protease production by P.CMBA and PMFS at 0.1 mmol. In this regard, PMSF sulfonates the essential serine residue in the active site and results in the complete loss of activity. This inhibition profile classifies this protease as serine hydrolases. The same results were confirmed by many authors (**Morihara, 1974; Bono *et al* 1996**). In addition, protease was found to be metal ion dependent in view of their sensitivity to metal chelating such as EDTA (**Dhandapani and Vijayaragavan, 1994**). Tween 80, the non-ionic detergent and surfactants inhibited the protease activity at 0.6 mmol.

CONCLUSION

In conclusion, a chemically defined medium suitable for the production of active, stable extracellular neutral protease activity by *Streptomyces microflavus* has been developed. The medium is solely constituted of water-soluble substances. The microorganism produces the highest level of proteolytic activity in excess of carbon sources and a relative deficiency of nitrogen supply. The secretion of activity depends upon careful control of the medium pH and efficient oxygen supply. *Streptomyces microflavus* synthesizes multiple proteases, serine and metal required proteases which offer an interesting potential for enzymatic and/or microbiological hydrolysis at industrial level. *Streptomyces microflavus* is a very promising strain for biotechnological application.

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العوامل التي تؤثر علي إنتاجية إنزيم البروتييز المتعادل المنتج من استربتوميسس ميكروفلافوس

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تم دراسة بعض العوام المؤثرة علي إنتاجية إنزيم البروتييـــز المتعـادل والمستخلص مـــن نـوع إستربتوميسس ميكروفلافـوس المعـزول من التربة المصرية.

تم استخدام بيئة النمو والتي تحتـوى علي النشا، نتـرات بوتاسيـوم ، ثنائـي البوتاسيوم الهيدورجين فوسفـات ، كبريتات مغنيسـوم ، كلوريد صوديوم ، كربونات كالسيوم وكبريتات الحديد.

وقد وجـد أن إنتاج إنزيم البروتييز يزيـد بنقص النسبـة بين حجـم البيئـة المستخدمة وبين الدورق المخروطي (٢٥٠ مللي) يكـون ٥٠ مللي (بنسبة ١: ٥) عند عـدد هزات ١٥٠ هزة في الدقيقة.

ويؤثـر الأس الهيدوروجينـي pH علـي إنتـاج الإنزيـم حيث وجد أنه يقل عند أكثر من pH5 وأقل من pH9 ونقصت كمية إنزيم البروتييز المتعادل بشدة عند درجة حرارة أكثر من ٣٠-٤٥م° وتم الحصول علـي أعلـى إنتاجيـة للإنزيـم بعد ثلاثـة أيام من التحضين.

وأوضحت الدراسـة أن إضافة النشا (٢٠جم/لتر) ومستخلص الخميـرة (٣,٨ جـم/لتر) كانت أفضـل مصادر الكربون والنتروجين علي الترتيب.

وأعطت أيضا كربونـات الكالسيـوم (٣جم/لتر) إنتاجيـة أعلـى من البروتييز وكـان ثنائي بوتاسيوم الهيـدروجين فوسفـات (١جم/لتر) أفضل مصـدر للفوسفات.

كمـا وجـد أن تركيـز (٠,١ مللي مول) مـن Parachloromercuribenzoic acid (P.CMBA) and Phenylmethylsulfonyl floride (PMSF)

قد أثر بشـدة علي نشاط إنزيم البروتييز المتعادل وقد أوقفت هذه المواد بالكامل نشاط الإنزيم عند هذا التركيز.

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