OPTIMIZATION OF CULTURE MEDIUM FOR ENHANCED PROTEASE BIOSYNTHESIS in *Streptomyces globisporus*

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ABSTRACT

In present study screening of strain strongly producing protease enzyme was conducted. *Streptomyces globisporus* 1/68 was found to possess maximum protein degrading capacity. The effect of carbon sources, inorganic and organic nitrogen sources on protease production by *Streptomyces globisporus* 1/68 was evaluated. The optimal carbon and nitrogen sources in the nutrient medium were selected. Maximal protease production was shown in medium with galactose as a carbon source (1.2 U/ml). Among inorganic nitrogen sources tested ammonium dihydrogen phosphate (NH₄H₂PO₄) supported moderate growth and protease production in *Streptomyces globisporus* 1/68 with enzymatic activity of 5.8 U/ml. The addition of organic nitrogen sources did not affect the protease biosynthesis in tested culture. Besides, amino acids supplemented in medium with selected nitrogen inorganic source had no significant effect on protease biosynthesis in by *Streptomyces globisporus* 1/68.

Keywords: Microbial Enzymes, Growth Conditions, Protease, Carbon Sources, Nitrogen Sources, *Streptomyces globisporus*.

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INTRODUCTION

Proteases are industrial enzymes that catalyze the hydrolysis of peptide bonds in proteins and have applications in detergent, food, feed, pharmaceutical, leather industries and bioremediation process¹⁻⁵. These enzymes account around 50-60% of the total worldwide enzyme market and can be obtained from plants, animals, and microorganisms⁶⁻¹⁰. However most commercially important proteases are obtained from microorganisms since microbial enzymes can usually be obtained in large quantities, on a regular basis and with the same quality. Besides, microbial enzymes are generally more stable, their production processes are faster and nutritional needs are relatively simple. In addition, microbes can be genetically manipulated to obtain the desired characteristics, to increase activity and productivity¹¹⁻¹³. The producers of proteases were found among *Aspergillus niger*, *A.terricola*, *A.oryzae*, *A.awamori*, *Bacillus cereus*, *Bacillus subtilis*, *Streptomyces griseus*, *Streptomyces chromogenes* *Actinomyces fradiae* etc.¹⁴⁻¹⁹ Recently, micromycetes and actinomycetes have been widely used because of the ease of their cultivation and high productivity.

Proteases produced by microorganisms are constitutive or inducible in nature, and their production depends on culture conditions. The ability to control the enzymes production by regulation of their biosynthesis allows not only to increase the yield of enzymes, but also to obtain enzyme preparations with certain properties. Various factors affect the production of the protease enzymes, such as nitrogen and carbon sources, the inoculum concentration, fermentation period, pH, temperature, etc.²⁰⁻²⁷ About 30% of the industrial enzymes cost depends on the cost of the nutrient medium. In this regards, the main objective of fundamental research for the industrial enzymes application is to reduce their production costs by optimization of fermentation medium. Present investigation focused on screening of microbial strain strongly producing protease enzyme and optimization of media components (carbon and nitrogen sources) for enhanced protease production by obtained strain.

EXPERIMENTAL

Material and Methods

The pure strains of *Aspergillus* and *Penicillium* fungi as well as *Streptomyces globisporus* 1/68 (own collection) were screened for extra cellular protease production. Primary selection was carried out on Chapek-Doks medium containing 1% sodium casein, peptone and wheat bran as specific substrates. The clearance zone and colony diameter were measured after 5 days of incubation time at 30°C. The ability of microorganisms to use specific substrate as the sole source of protein was studied. Only positive and better zone formed strain was taken for further study.

The effect of various carbon and nitrogen sources on protease production was investigated. Basal medium of the following composition was used: NaNO₃ - 0,15% (on N); KCl - 0,05%; MgSO₄ - 0,05%; KH₂PO₄ - 0,1%; FeSO₄ - 0,001%; mannitol - 2%.

As inorganic nitrogen sources (NH₄)₂SO₄, NH₄Cl, NH₄NO₃, NaNO₃, NH₄H₂PO₄, (NH₄)₃PO₄, (NH₄)MoO₄ at a concentration of 0.5% by volume of the medium were used.

As organic nitrogen sources Peptone, Casein, Wheat bran, Wheat flour, Soy flour, Whey 2%, Whey 5% at a concentration of 1.0% by volume of the medium were used.

As carbon sources Galactose, Arabinose, Fructose, Starch, Maltose, Mannitol, Sucrose, Glucose, Mannose, Glycerin, Lactose and Sorbitol were used at a concentration of 1.5% by volume of the medium.

A 2% microbial suspension was sterilized in all flasks and placed on a shaker at 220-240 rpm for 72 hours. After this fermentation time, the protease activity of the culture fluid of all variants was assayed according to Anson's method²⁸. One unit of the protease activity was expressed in micromoles of tyrosine formed in 1 ml of culture fluid for 1 min.

Microbial biomass was determined by gravimetric method. Mycelium was separated from the medium by filtration through Whatman filter paper, washed, dried to constant weight at 86°C. All the analyses were performed in triplicate, and the results were expressed as mean ± standard deviation using Excel 2010.

RESULTS AND DISCUSSION

Screening of Microscopic Strain Strongly Producing Protease Enzyme

To assess the potential for protease production *Aspergillus* and *Penicillium* fungi as well as *Streptomyces globisporus* 1/68, known as potential protease producers were analyzed for protease activity. Microbial cultures were inoculated in agar medium containing protein substrates. The zone of clearing around the microbial growth was indicator of protease production (Fig.-1).

Fig.-1: Zones of Substrate Hydrolysis

The clear zone formation concerns the ability of colonies with confirmed protein hydrolysis. During the initial screening of pure strains of *Aspergillus* and *Penicillium* fungi as well as *Streptomyces globisporus* 1/68 for synthesis of protease enzyme it was found that not all strains grew on the medium containing casein, peptone and wheat bran as specific substrates (Table-1).

Table-1: Extracellular Protease Enzyme Production on Various Protein Substrates			
Strain	Zones of Substrate Hydrolysis (mm)		
	Casein	Peptone	Wheat Bran
<i>Streptomyces globisporus</i> 1/68	25.6	22.5±1.2	21.4±1.2
<i>Aspergillus awamori</i> 16	19.6±1.2	17.5±1.6	15.4±0,9
<i>Aspergillus awamori</i> 22	16.9±1.3	15.4±1.3	14.3±1.8

<i>Aspergillus awamori</i> 21/95	15.5±1.4	13.4±1.6	11.3±1.7
<i>Aspergillus awamori</i> 21/96	16.7±1.3	15.6±0.8	12.1±1.5
<i>Aspergillus awamori</i> 426	11.3±1.2	13.2±1.2	13.3±1.1
<i>Aspergillus foetidus</i> 26	0	0	0
<i>Aspergillus oryzae</i> 3-9-15	0	0	0
<i>Aspergillus oryzae</i> 476	0	0	0
<i>Aspergillus niger</i> II	0	0	0
<i>Penicillium chrysogenum</i> 241	0	0	0
<i>Penicillium chrysogenum</i> 245	0	0	0

Among the studied strains, *Streptomyces globisporus* 1/68 was found to possess maximum protein degrading capacity which was identified by the presence of clearance zone in protease screening medium containing sodium casein, peptone and wheat bran as substrate. Zones of substrate hydrolysis by *Streptomyces globisporus* 1/68 on the 5th day were 25.6 mm. In other variants, the diameter of clearance zones varied from 11.3mm to 16.9mm. Thus, *Streptomyces globisporus* 1/68 was selected for further investigations.

Effect of Various Inorganic and Organic Nitrogen Sources on Protease Production by *Streptomyces globisporus* 1/68

The composition of the growth medium is very important aspect in the development of fermentation processes. Nitrogen is one of the essential elements, which play an important role in physiological control, regulation of metabolisms and enzyme synthesis. In present work the effect of different inorganic and organic nitrogen sources on protease production by *S. globisporus* 1/68 was investigated. The effect of nitrogen sources on protease production is presented in Tables-2 and 3.

Table-2: Effect of Inorganic Nitrogen Sources on Protease Production by *Streptomyces globisporus* 1/68

Nitrogen Source	pH	Biomass, g/100 ml	Protease Activity, U/ml
(NH ₄) ₂ SO ₄	4.0	0.143±0.08	1.4±0.5
NH ₄ Cl	4.0	0.158±0.10	2.1±0.38
NH ₄ NO ₃	6.0	0.134±0.11	3.2±0.44
NaNO ₃	6.5	0.243±0.08	0.9±0.36
NH ₄ H ₂ PO ₄	6.4	0.103±0.09	5.8±0.49
(NH ₄) ₃ PO ₄	5.9	0.14±0.10	0
(NH ₄)MoO ₄	6.6	0.139±0.08	2.7±0.61

Table-3: Effect of Organic Nitrogen Sources on Protease Production by *Streptomyces globisporus* 1/68

Nitrogen Source	pH	Biomass, g/100 ml	Protease Activity, U/ml
Peptone	5.7	-	1.6±0.33
Casein	5.0	-	0.7±0.45
Wheat bran	6.3	-	1.1±0.4
Wheat flour	5.3	0.135±0.08	1.5±0.29
Soy flour	4.7	0.186±0.07	1.9±1.02
Whey 2%	5.8	0.364±0.34	0.9±0.65
Whey 5%	6.0	0.112±0.09	1.6±0.77

The experiments showed that a significant amount of protease enzyme was formed on inorganic nitrogen sources in comparison with organic sources. Among the inorganic nitrogen sources, NH₄H₂PO₄ supported moderate growth and protease production in *Streptomyces globisporus* 1/68 with enzymatic activity of 5.8 U/ml. All other nitrogen and organic sources used had less effect on protease production. This indicates that NH₄H₂PO₄ is the most efficient source for protease production by *Streptomyces globisporus* 1/68.

As *Streptomyces globisporus* 1/68 is actinomycete it was supposed that amino acids can affect the protease production in *Streptomyces globisporus* 1/68. Therefore, the effect of amino acid supplemented in medium with an optimal nitrogen inorganic source (NH₄H₂PO₄) was investigated. As can be seen from data presented in Table-4 all amino acids supplemented in medium with nitrogen

inorganic source ammonium dihydrogen phosphate had no effect on protease production in *Streptomyces globisporus* 1/68.

Table-4: Effect of Amino Acids on Protease Production by *Streptomyces globisporus* 1/68

Nitrogen Source	Amino Acid	pH	Biomass, g/100 ml	Protease Activity, U/ml
NH ₄ H ₂ PO ₄	-	6.4	0.103±0.04	5.8±0.34
NH ₄ H ₂ PO ₄	Hydrochloric arginine	6.5	0.146±0.09	1.1±0.45
NH ₄ H ₂ PO ₄	Arginine hydrochloride	5.4	0.102±0.1	1.2±0.62
NH ₄ H ₂ PO ₄	Valine	5.6	1.132±0.23	0
NH ₄ H ₂ PO ₄	Histidine	6.0	0.084±0.01	2.0±0.32
NH ₄ H ₂ PO ₄	Glutamine	5.4	0.05±0.02	2.8±0.19
NH ₄ H ₂ PO ₄	Leucine	5.1	0.253±0.1	0.9±0.21
NH ₄ H ₂ PO ₄	Hydrochloric acid lysine	5.6	0.032±0.01	1.1±0.18
NH ₄ H ₂ PO ₄	Lysine monochloride	5.4	0.061±0.02	2.7±0.52
NH ₄ H ₂ PO ₄	Methionine	6.0	0.082±0.03	2.1±0.49
NH ₄ H ₂ PO ₄	Ornithine	5.5	0.093±0.01	1.2±0.44
NH ₄ H ₂ PO ₄	Threonine	5.7	0.156±0.02	1.1±0.36
NH ₄ H ₂ PO ₄	Tryptophan	5.4	0.160±0.07	0
NH ₄ H ₂ PO ₄	Serine	4.5	0.105±0.07	1.9±0.28
NH ₄ H ₂ PO ₄	β-phenyl-α-alanine	5.7	0.099±0.04	1.1±0.48

There are various ways of regulation of enzyme biosynthesis. In some cases much depends on whether the enzyme is constitutive or inducible. An inducible enzyme is an enzyme that is produced under conditions where it has no adaptive properties (for example, in the presence of an inducer/substrate in a nutrient medium), in contrast to a constitutive enzyme, which is produced all the time²⁹.

Therefore, next step of study was to elucidate the constitutive or inducible nature of protease enzyme produced by *Streptomyces globisporus* 1/68. To identify the induced or constitutive nature of protease biosynthesis, an experiment was conducted with simultaneous addition of protein substrates in a production medium (Table-5).

Table- 5: Effect of Protein Substrates on the Protease Biosynthesis by *Streptomyces globisporus* 1/68

Variant	pH	Biomass, g / 100 ml	Protease Activity, U/ml
NH ₄ H ₂ PO ₄	6.4	0.103±0.14	5.8
NH ₄ H ₂ PO ₄ +Peptone	5.0	0.111±0.31	5.3
NH ₄ H ₂ PO ₄ +Casein	5.3	0.238±0.45	0.5
NH ₄ H ₂ PO ₄ + Wheat bran	5.4	0.304±0.23	1.1
NH ₄ H ₂ PO ₄ + Milk whey 5%	6.0	0.156±0.09	3.0
NH ₄ H ₂ PO ₄ + Soy flour	5.2	0.344±0.46	2.6
NH ₄ H ₂ PO ₄ + Wheat flour	5.5	0.121±0.12	2.5
NH ₄ H ₂ PO ₄ + Methionine	4.6	0.323±0.31	1.6

According to our results obtained, protease biosynthesis was not stimulated by the presence of protein substrates in the culture medium. This indicates that the production of the protease by *Streptomyces globisporus* 1/68 is not inducible.

Effect of Various Carbon Sources on Protease Production by *Streptomyces globisporus* 1/68

Carbohydrates are one of the important factors in fungal growth. Effect of various carbon sources on protease production in *Streptomyces globisporus* 1/68 is shown in Table-6. The protease activity ranged from 0.62 U/ml to 1.2 U/ml.

According to the obtained experimental data, all carbon sources can be divided into three groups. Monosaccharides and starch are the most digestible carbon sources, giving biomass accumulation from 0.7 to 1.1 g/100 ml and protease activity from 0.75 to 1.2 U/ml. The second group of carbon

sources includes sucrose, glucose, mannose, and glycerol, which gave biomass accumulation from 0.475 to 0.6 g/100 ml. The biosynthesis of protease enzyme by *Streptomyces globisporus* 1/68 was maximal when glucose was used as a carbon source (1.0 U/ml). The third group of carbohydrates includes sorbitol and lactose, which are poorly digestible carbohydrates. These carbohydrates provided a weak biomass accumulation – from 0.15 to 0.295 g/100ml.

Thus, *Streptomyces globisporus* 1/68 assimilated mono-, di-, polysaccharides. Among them, galactose, glucose, maltose and starch provided relatively good growth and enzyme production, up to 1.1-1.2 U/ml.

Table-6: Effect of Organic Nitrogen Sources on Protease Production by *Streptomyces globisporus* 1/68

Carbon Source	pH	Biomass, g/100 ml	Protease Activity, U/ml
Galactose	5.5	1.1±0.4	1.2±0.92
Arabinose	5.5	0.9±0.12	0.75±0.77
Fructose	5.6	0.89±0.32	0.82±0.58
Starch	5.8	0.8±0.23	1.1±0.45
Maltose	6.0	0.7±0.11	1.1±0.65
Mannitol	6.5	0.69±0.33	0.67±0.7
Sucrose	5.4	0.61±0.42	0.72±0.45
Glucose	5.0	0.6±0.32	1.0±0.65
Mannose	5.5	0.55±0.06	0.77±0.32
Glycerol	5.0	0.475±0.1	0.75±0.88
Lactose	5.5	0.295±0.09	0.62±0.74
Sorbitol	5.6	0.15±0.08	0.7±0.61

CONCLUSION

The composition of the medium is the most important aspect to be considered when growing microorganisms. The culture medium should include all essential nutrients that microorganism requires. The protease production has been affected by a variety of physical and chemical factors, such as the composition of the growth medium, cell growth, methods of cultivation, inoculum concentration, time of incubation, pH, temperature, carbon, nitrogen and mineral sources, etc. Various physical and chemical factors such as composition of growth medium, concentration of inoculum, incubation time, pH, temperature, carbon and nitrogen sources have effect on protease biosynthesis. Our results indicate that some cultivation conditions such as carbon and nitrogen sources play significant role on protease biosynthesis by *Streptomyces globisporus* 1/68. The results indicated that among the various carbon sources galactose, glucose, maltose, and starch are the most suitable carbon sources for maximal protease biosynthesis (1.0-1.2 U/ml). Among the various inorganic and organic nitrogen sources $\text{NH}_4\text{H}_2\text{PO}_4$ supported moderate growth and protease production in *Streptomyces globisporus* 1/68 with enzymatic activity of 5.8 U/ml. Thus, nitrogen and carbon sources are very important in metabolism of microorganisms especially in the enzymes biosynthesis.

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