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SCREENING OF A HIGH GLUTAMINOLYTIC ENZYME PRODUCING STRAIN AND ITS EXTRACELLULAR PRODUCTION BY SOLID STATE FERMENTATION

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ABSTRACT

This study presents new and alternative fungal strains for the production of glutaminolytic enzyme which has great potential to use in industrial and biotechnological processes. Forty fungal strains isolated from food and other sources were screened using minimal glutamine agar containing L-glutamine and phenol red as indicator for glutaminase production. All the strains were positive for glutaminase activity in a rapid-plate assay, as evidenced by pink halos. The cultivation of the best strain (*Trichoderma harzianum*) by solid state fermentation resulted in high quantities of extracellular glutaminolytic enzyme (88 U/g) with a zone of 1.2 cm when cultured at 30 °C for 5 days.

KEYWORDS

Solid state fermentation, Screening, Glutaminolytic enzyme and Fungi.

INTRODUCTION

The enzyme L-glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which generates L-glutamic acid and ammonia from L-glutamine. The L-glutaminase is the cellular enzyme deaminating L-glutamine and acts as proteolytic endopeptidase, which hydrolyses the peptide bonds present in the interior of the protein molecules. A variety of microorganisms, including bacteria, yeast, moulds and filamentous fungi, have been reported to produce L-glutaminase [1, 2, 3] of which the most potent producers are fungi [4]. On an industrial scale, glutaminases are produced mainly by *Aspergillus* and *Trichoderma* spp. [5, 6, 7].

The action of glutaminase plays a major role in the cellular nitrogen metabolism of both, prokaryotes and eukaryotes [1, 8]. In recent years, L-glutaminase has received attention as a therapeutic against cancer and HIV [9, 10], as a bio-sensing agent in monitoring glutamine levels [1, 11], for the production of specialty chemicals like theanine by c-glutamyl transfer reactions and as a flavour enhancer in food industry[12]. The use of L-glutaminase as a flavour- enhancing agent in Chinese foods has replaced the use of monosodium glutamate which acts as an allergen for individuals [6, 13, 14, 15].

In the search for fungal glutaminases, it is thought that if extracellular glutaminases are able to be found, they will be produced more easily by solid state fermentation using agro industrial residues, in order to meet the growing industrial demands.

In our present investigation, a variety of fungal cultures were tested for their extracellular enzyme productivity. The study also focuses on the pH and dye-based fast procedure for potential glutaminase producers.

MATERIALS AND METHODS

Microorganism

Forty fungal species used in the study were isolated from various substrates like soya sauce, fungal infected coconut (wet kernel) and spoiled bread[16]. The fungal cultures were maintained on potato-dextrose agar, incubated at 30 °C for 7 days and then stored at 4 °C until use.

Screening for glutaminolytic activity

Screening of fungal isolates was carried out using minimal glutamine agar (MGA) method described by El-Sayed[7] and Gulati *et al.,* 1997[17] with some modifications. Components of MGA (gram/litre) include 0.5 Dextrose; 0.5 KCl; 0.5 $MgSO_4$; 1.0 KH_2PO_4; 0.1 $FeSO_4$; 0.1 $ZnSO_4$; 25 NaCl; 10 L-glutamine; 0. 25 phenol red in which L-glutamine act as carbon and nitrogen source and phenol red act as pH indicator. Fungal colonies were inoculated on the prepared plates and incubated at 30 °C for 48 hours. One un-inoculated plate was kept as control. After incubation, L-glutaminase activity was reported based upon intensity of produced pink color and pink zone diameter.

Fermentation medium

Five grams of substrate (wheat bran) were dispensed into 250 mL Erlenmeyer conical flasks and moistened with 10 mL of salt solution containing glucose 0.6%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.05% and KCl 0.05%. The flasks were autoclaved at 121 °C for 25 min, cooled to room temperature and inoculated with 2 mL of the fungal conidial suspension (10^6 spores/ml). The inoculated flasks were mixed thoroughly and incubated at 30 °C for 5 days.

Crude enzyme extraction

The crude L-glutaminase was extracted from the fermented solid substrate by using

citrate-phosphate buffer (pH 7.0). The fermented solid substrate was mixed thoroughly with 50 mL of the buffer (pH 7.0) using a rotary shaker 200 rpm for 30 min. The entire contents of the flask were squeezed through a cheese cloth, the pooled extract were centrifuged at 4 °C for 20 min at 10000 rpm.

Estimation of enzyme activity

L-glutaminase was assayed by direct Nesslerization method according to Imada *et al*. [18]. The enzymatic reaction mixture contains 1 mL of 1% L-glutamine in citrate-phosphate buffer (pH 7.0) and 1 mL of the crude enzyme incubated at 30 °C for 1 hour. The enzymatic activity was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid. The reaction mixture was centrifuged at 5000 rpm for 5 min to remove the precipitated protein. Then 0.1mL of above mixture was taken and added to 3.7 mL of distilled water. Then 0.2 mL Nessler's reagent was added to it, after 15 min the developed color was measured at 480 nm using a spectrophotometer [19]. Enzyme and substrate blanks were used as controls. The ammonium concentration of the reaction was determined by inference from the standard curve of ammonium sulphate. One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1µ mol of ammonia under optimal assay conditions.

Protein estimation

The concentration of protein of the crude enzyme was estimated by Folin reagent according to the protocol of Lowry et al.,[20] using bovine serum albumin as standard. The protein concentration was expressed by mg/ml of crude enzyme.

RESULTS AND DISCUSSION

A total number of 40 isolates of fungi were isolated and identified as genera of *Aspergillus* (15), *Acremonium* (1), *Fusarium* (3), *Rhizopus* (1), *Mucor* (2), *Penicillium* (6), *Trichoderma* (4) and Basidiomycetes (7). Most frequently isolated genus was *Aspergillus* (Table 1).

All the isolated strains were screened for its L-glutaminase production. This screening of filamentous fungi is based on the semi qualitative method described by Gulati *et al*., 1997 [17]. The plate assay used for screening the isolates to determine their glutaminase activity (equal to diameter of the pink zone) is also an indication of the amount of glutaminase produced by the colony or isolate. The different isolates exhibited pink zones around them. The size or diameter of these zones was proportional to the glutaminase produced by the colony. The colour change of the medium from yellow to pink is an indication of the extra cellular L-glutaminase production by the colony. This colour change is due to change in the pH of the medium, as L-glutaminase causes the breakdown of amide bond in L-glutamine and liberates ammonia. Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around the microbial colonies producing L-glutaminase.

It was proposed that the strain *Trichoderma harzianum* produced a zone of 1.2 cm. Similar results were those in agreement with various workers [7, 21, 22]. In SSF, the selection of a suitable solid substrate plays a crucial role for the fermentation process. Solid substrates utilized in solid state fermentation processes are generally insoluble in water and provides nutrients for the growth of microbial culture and anchorage for the growing cells. Unlike bacteria and yeasts that grow by surface adhering to solid substrates, filamentous fungi have the potentiality to deeply penetrate the solid particles for nutrient up taking.

In the present investigation, wheat bran has been selected as substrate because of its particle size and agglomeration properties. However, all the 40 isolates were employed for glutaminase production by solid state fermentation and the results were reported in Table 1. This revealed that *Trichoderma harzianum* had the maximum glutaminase activity (88 U/g) followed by *Aspergillus flavus* which produced a zone of 1.4 cm and activity of 86 U/g. El-sayed reported a production of

24.13 U/gds using *Trichoderma koningii* [7]. As expected, isolates which exhibited a pink zone with maximum diameter also produced the maximum amount of glutaminase by solid state fermentation.

Table 1

L-glutaminase enzyme production by selected isolates.

S.No	Name of the Isolates	Zone Diameter (cm)	Protein concentration (mg/ml)	Glutaminase activity (U/g)
1.	*Aspergillus niger*	0.20	0.20	18
2.	*Aspergillus flavus*	1.40	0.10	86
3.	*Aspergillus terreus*	0.30	0.40	24
4.	*Aspergilllus flavipes*	0.45	0.30	36
5.	*Aspergillus ornatus*	0.20	0.20	25
6.	*Aspergillus erythrocephalus*	0.35	0.35	36
7.	*Aspergillus sydowii*	0.40	0.60	44
8.	*Aspergillus oryzae*	0.40	0.40	40
9.	*Aspergillus andulaceus*	0.45	0.55	44
10.	*Aspergillus glaucus*	0.36	0.45	42
11.	*Aspergillus alutaceus*	0.32	0.40	36
12.	*Aspergillus versicolor*	0.80	0.45	60
13.	*Aspergillus wentii*	0.70	0.65	58
14.	*Aspergillus fumigatus*	0.50	0.80	60
15.	*Aspergillus japonicum*	0.20	0.40	28
16.	*Acremonium forcatum*	0.20	0.20	22
17.	*Humicdo insolens*	0.40	0.25	32
18.	*Fusarium oxysporum*	0.30	0.30	36
19.	*Fusarium solani*	0.20	0.30	26
20.	*Fusarium dimerum*	0.10	0.35	24
21.	*Mucor circinelloides*	0.90	0.85	68
22.	*Rhizopus oryzae*	0.60	0.55	54
23.	*Rhizomucor meihei*	0.45	0.60	56
24.	*Penicillium chrysogenum*	1.05	0.85	74
25.	*Penicillium digitatum*	0.35	0.60	34
26.	*Penicillium citrinum*	0.30	0.40	28
27.	*Penicillium brevicompactum*	0.20	0.20	12
28.	*Penicillium notatum*	0.20	0.20	18
29.	*Penicillium restrictum*	0.30	0.80	42
30.	*Trichoderma species*	0.30	0.60	58
31.	*Trichoderma harzianum*	1.20	1.20	88
32.	*Trichoderma reesei*	0.40	0.60	42
33.	*Trichoderma viride*	0.60	0.60	69
34.	*Basidiomycetes species* I	0.20	0.08	14
35.	*Basidiomycetes species* II	0.20	0.15	20
36.	*Basidiomycetes species* III	0.30	0.20	24
37.	*Basidiomycetes species* IV	0.30	0.25	24
38.	*Basidiomycetes species* V	0.80	0.40	36
39.	*Basidiomycetes species* VI	0.40	0.20	12
40.	*Basidiomycetes species* VII	0.60	0.15	22

CONCLUSION

Economically this enzyme could be produced from cheap, untreated, agro-industrial residues. The yield obtained in the present investigation would have to be further increased for its industrial importance, as it has proved solid state fermentation process as a prospective technique for the large-scale production of microbial metabolites of biotechnological and medical importance.

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