OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF L-GLUTAMINASE WITH MIXED SUBSTRATE BY SOLID STATE FERMENTATION USING Aspergillus wentii MTCC 1901

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Abstract

L-Glutaminase, an amidohydrolase enzyme has been a choice of interest in the treatment of leukaemia since the discovery of its anti-tumour properties. Because it is a potent anti leukemic agent and a flavor-enhancing agent used in the food industry, many researchers have focused their attention on L-glutaminase. L-Glutaminase is majorly produced by micro organisms including bacteria, yeast and fungi. In the present study, production of L-Glutaminase from fungi, Aspergillus wentii was reported. Solid state fermentation was followed in the study for the enzyme production using different agro-industrial by products which include coconut oil cake, sesame oil cake, groundnut oil cake and neem oil cake. Out of them potential substrates were screened and used as mixed substrate. Mixed substrate was selected by mixing coconut oil cake and sesame oil cake in different compositions. The best composition, 1.25gm coconut oil cake and 3.75gm sesame oil cake was selected. Effect of process parameters namely temperature, pH, incubation time, moisture content, inoculum volume on enzyme production was investigated. Also effect of supplementary carbon sources, nitrogen sources, metal ions and glutamine concentration was studied and their optimum conditions were determined. The organism produced high levels of enzyme at an optimum temperature of 28 °C and optimum pH 7.0, after 120h of incubation with 40% inoculum volume and 50% moisture content. Enhanced production was obtained on addition of 1% W/V D-glucose, peptone, magnesium sulphate and 1% W/W glutamine as supplements which showed an increase to four folds. Using this optimized media components and parameters; the L-Glutaminase activity 496U/gds was obtained.

Keywords: L-Glutaminase, leukaemia, Aspergillus wentii, solid state fermentation, mixed substrate.

1. INTRODUCTION:

L-glutaminase(L-glutamine amidohydrolase E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine to L-glutamic acid and ammonia. This is an essential enzyme for the synthesis nitrogenous metabolic of various intermediates. Glutaminase, synthesized by various bacteria, fungi, yeast, moulds and filamentous fungi catabolises glutamine in micro-organisms. Mammalian cells also synthesis this enzyme which is involved in the generation of the energy using glutamine as the major respiratory fuel. Thus, many types of tumour cells as well as actively dividing normal cells exhibit high rates of glutamine utilization [7].

In recent years glutaminase has attracted much attention with respect to proposed applications in pharmaceuticals as anti-leukemic agent [19] and in food industry as flavor enhancing agent [20]. L-glutaminase in combination with or as an alternative to asparaginase could be of significance in enzyme therapy for cancer especially acute lymphocytic leukaemia [19]. Its commercial importance as anticancer and flavor enhancing agent demands not only the search for better yielding viable strains, but also economically viable bioprocesses for its large scale production [14]. Another important application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures [15]. Marine microorganisms hold significance in food industry by virtue of their ability to produce salt tolerant L-glutaminase, as salt and thermo tolerant glutaminases are needed in soy sauce fermentation [4].

Over the last two decades, SSF has gained significant attention for the development of industrial bioprocesses, particularly due to lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination. In addition, it is ecofriendly, as it mostly utilizes solid agro-industrial wastes (resides) as the substrate (source of carbon).

Oil cakes rich in fibre, protein and energy content offer potential benefits when used as substrate in developing bioprocesses for the production of industrial enzymes. The bioprocess utilizing oil cakes has been attractive due to relatively cheaper availability of the oil cakes throughout the year making it favourable when economics is considered [18].

Mixed substrate fermentation has been more advantageous for the production of enzymes than single substrate fermentation [18],[16]. In the present study L-glutaminase was produced using mixed substrate by solid state fermentation. Oil cakes were used as the substrates. Later the effects of various process parameters and inducers have been studied and they were optimized for increasing the enzyme yield.

2. MATERIALS AND METHODS

2.1 Substrate

Four different cheap agro residues namely Groundnut oil cake (GOC), Coconut oil cake (COC), Sesame oil cake (SOC) and Neem oil cake (NOC) were collected from a local market of Rajam, India. The cakes were sun dried and ground to fine powder.

2.2 Microorganism

Aspergillus wentii MTCC 1901 obtained from Microbial Type Culture Collection, Chandigarh, India was used throughout the study.

2.3 Growth Medium and Growth Conditions

The culture was maintained on PDA agar slants having the composition (g/L): Potatoes infusion from 200, Dextrose 20 and agar-agar 15 with pH 5.6 ± 0.2 . 39 gm of PDA medium and 0.1 gm agar-agar were weighed in 1000ml distilled water and used as growth medium. The culture was incubated at 28°C for 120h. Sub culturing was carried out once in every 15 days and the culture was stored at 4°C.

2.4 Inoculum Preparation

The inoculum was prepared by scrapping the spores of the fungi, *Aspergillus wentii* using Tween 80 solution.

2.5 Microbial Fermentation

5g of each substrate was weighed in 250ml Erlenmeyer flasks separately. The substrates were moistened with 2ml of moistening medium (distilled water used here) and were autoclaved at 121°C (15 lb) for 20 min, cooled to room temperature and then inoculated with 2ml of inoculum. The inoculated flasks were incubated at 28°C in an incubator for 120h.

2.6 Mixed Substrate Composition

Two potential substrates Coconut oil cake and Sesame oil cake were screened out of four and mixed in different compositions according to mixture design. High yield was reported when 1.25gm of coconut oil cake and 3.75gm of sesame oil cake were mixed. Hence the mixed substrate composition 1.25:3.75 proved to be the best composition.

2.7 Enzyme Extraction

After the incubation period, the crude enzyme from the fermented substrate was extracted using 0.1M phosphate buffer (pH 8). After mixing the fermented substrate with 41 ml of phosphate buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was filtered and

the filtrate was centrifuged at 10,000 rpm for about 10 min at 4° C in a cooling centrifuge. Supernatant was collected and used for enzyme assay.

2.8 Enzyme Assay

The activity of L-glutaminase was determined by estimating the amount of ammonia liberated [6].

2.9 Optimization of Process Parameters

The strategy adopted was to optimize one particular parameter at a time and then include it at its optimum value in the next optimization step. The parameters optimized were: temperature, pH, incubation time, moisture content, inoculums volume, carbon source, nitrogen source, metal salts and glutamine concentration.

3. RESULTS AND DISCUSSIONS

3.1 Substrate Selection

Among all the substrates, the maximum glutaminase yield was observed for sesame oil cake (68.33U/gds) followed by coconut oil cake (47.83U/gds) suggesting their importance in selecting them as agro residues for glutaminase production as shown in Chart-1. The lowest production was observed with neem oil cake (20.5U/gds) followed by ground nut oil cake (27.33U/gds).

3.2 Mixed Substrate Composition

For determining the substrate composition, experiment was done with five combinations of sesame oil cake and coconut oil cake. The results showed that glutaminase production varied within the range of 47.83U/gds to 123U/gds. The highest amount of enzyme yield (123U/gds) was observed when the substrates were mixed in 1.25:3.75 ratio which contained 1.25gm of coconut oil cake and 3.75gm of sesame oil cake for 5gm of total substrate.



Substrates



3.3 Effect of Temperature

Incubation was carried out at different temperatures ranging from 18°C to 58°C among which the maximum glutaminase yield was observed at 28°C (123U/gds) which seemed to be the optimum temperature (Fig-1). Similar observations were reported for glutaminase from *Streptomyces avermitilis* and *Streptomyces labedae* [1].



Fig-1: Effect of temperature on glutaminase production

3.4 Effect of pH

pH of the extraction buffer plays a key role in the change in the amounts of enzyme yield. The pH of the buffer was varied from 5 to 9 and the maximum yield was reported at pH 7.0 which is 150.33U/gds (Fig-2). The result obtained was in agreement with *T.koningii* where pH 7.0 was favourable for high enzyme yield [5].



Fig-2: Effect of pH on glutaminase production

3.5 Effect of Incubation Time

Incubation period was the most important physical variable in solid state fermentation. To determine the optimum incubation period, fermentation flasks were incubated for different time duration (3 to 7 days). Enzyme assay was done after every 24 h and maximum enzyme production was observed to be 150.33U/gds on 5th day of incubation (Fig-3). Further incubation did not show increment in enzyme yield. These results are similar to *T.koningii* [2] and *A.flavus* [12].



Fig-3: Effect of incubation time on glutaminase production

3.6 Effect of Moisture Content

The substrate was moistened with different amounts of distilled water ranging from 1ml to 3 ml i.e., 20% to 60%. The maximum yield (177.66U/gds) was observed with 50% moisture content (Fig-4). In SSF, microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents [13]. A reduction in the solubility of nutrients of the substrate and a low degree of swelling are the disadvantages of low moisture content. Similar results were reported by *A.flavus* [12].



Fig-4: Effect of moisture content on glutaminase production

3.7 Effect of Inoculum Volume

The amount of inoculum plays a major role in the production of the enzyme. Varied amounts of inoculum were added to the substrate ranging from 0.5ml to 2.5ml i.e., 10% to 50%. Among them fermentation flask inoculated with 40% i.e., 2ml inoculum reported highest yield 177.66U/gds (Fig-5). A low inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass and deplete the substrate of nutrients or accumulation of some non-volatile self inhibiting substances inhibiting the product formation [10].With the optimum inoculum concentration, there is a balance between the proliferating biomass and availability of nutrients that supports maximum enzyme production. These results correlate with the results of *T.koningi* [2].



Fig-5: Effect of inoculum volume on glutaminase production

3.8 Effect of Carbon Sources

Extracellular enzyme production greatly depends on the composition of the medium. The addition of carbon sources influences the biosynthesis of L-glutaminase production. When 1% W/V of different carbon sources were added to the substrate the yield has increased slightly. Among them glucose reported high amounts of enzyme yield 307.5U/gds (Fig-6). Similar results were observed for the effect of carbon source where glucose showed high yield for actinomycetes [17], *B.diminuta* [8], *Streptomyces sp.-SBU1* [11], *Streptomyces avermitilis* and *Streptomyces labedae* [1].

3.9 Effect of Nitrogen Sources

Selection of best nitrogen sources can be an important limiting factor in the microbial production of enzymes [3]. Different nitrogen sources of 1% W/V were added to the medium out of which peptone showed high amounts of yield

355.33U/gds (Fig-7). Similar results were reported by *B.diminuta* [8] and *Streptomyces labedae* [1] where peptone showed high enzyme yield.

3.10 Effect of Metal Ions

Metal ions were added to enhance the production. Interestingly all metal ions increased the glutaminase yield. The maximum enzyme yield was reported with MgSO₄, 430.6U/gds (Fig-8). This suggests that metal ions are detrimental to the improvement of glutaminase production as the metals might act as cofactors for many enzymes involved in intermediatory metabolism [9].Similar results were also reported for glutaminase production with *A.flavus* [12].



Fig-6: Effect of carbon sources on glutaminase Production



Fig-7: Effect of nitrogen sources on glutaminase production



Fig- 8: Effect of metal ions on glutaminase production

3.11 Effect of Glutamine

Since L-Glutamine is the substrate of L- Glutaminase, the addition to fermentation medium stimulates the enzyme production. It also serves as source of energy and carbon. L-Glutamine concentration needed for maximal glutaminase yield was similar irrespective of the nature of the substrates used. Enzyme yield was higher with an optimum at 1% W/W glutamine concentration which is 492U/gds (Fig-9).



Fig-9: Effect of glutamine concentration on glutaminase production

4. CONCLUSION

This study has been done with a view of exploring the possibility of using coconut oil cake and sesame oil cake as mixed substrate for the production of L-Glutaminase. Also the strain *Aspergillus wentii* showed potential results to

produce L-Glutaminase and can be useful for industrial production of the enzyme. The optimized process parameters enhanced the production to four folds which was reported to be 492U/gds.

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