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Production and Optimization of L-Glutaminase with Mixed Substrate using Aspergillus Wentii MTCC 1901 by Solid State Fermentation

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***Abstract*— Extracellular L-Glutaminase, a hydrolytic enzyme was produced by solid state fermentation using Aspergillus Wentii MTCC 1901 with mixed substrate. Among Manila Tamarind (Pithecellobium dulce), Palmyra Tuber (Borassus flabellifer) and Tapioca (Manihot esculenta), Manila Tamarind Powder and Palmyra Tuber powder were selected and mixed in different ratios for fermentation medium. The maximum yield was obtained at the ratio of 3.5:1.5 (Manila Tamarind : Palmyra Tuber). Different parameters optimization processes were investigated on SSF namely incubation time (144hrs), temperature (30^{0}C), pH (6.8), and moisture content (45%), inoculum volume (2ml) and also supplemented with carbon sources, nitrogen sources, metal ions and glutamine concentration. After optimization, the productivity of L-Glutaminase obtained maximum yield of 703.8 U/gds showed an increase of 3.8 fold .The study indicates mixed substrates of agro residues Manila Tamarind Powder and Palmyra Tuber powder were more effective for the production of L-Glutaminase using SSF.**

***Keywords*— *L-Glutaminase, Aspergillus Wentii, Borassus flabellifer, Pithecellobium dulce, soid state fermentation*.**

I. INTRODUCTION

Enzymes are macromolecular biocatalysts produced by the living cells to bring about highly specific biochemical reactions forming parts of the metabolic process of the cells. Microbial enzymes are economic and environmental friendly. Use of enzymes as drug is a crucial facet of today's pharmaceutical industries.

In biomedical sciences emphasize the involvement of the enzyme L-Glutaminase (EC.3.5.1.2) is a cellular hydrolytic enzyme that generates Glutamic acid and ammonia from Glutamine. Glutaminase plays a major role in nitrogen metabolism of both prokaryotes and eukaryotes [1]. In the past few decades, eminent attention has been paid on certain principles that could serve as guides in the utilization of enzyme for the treatment of neoplasms have been suggested [2]. Intrest on the amidohydrolases started with the discovery of their anti-leukaemic properties and a lot of efforts have gone in to extentive studies on microbial L-Glutaminase with the intention of developing them as a potent anti-leukaemic agent and against HIV [3,4,5] .

Unlike normal cells, leukemic cells do not demonstrate L-Glutamine synthatase and thus depend on the exogenous supply of L-Glutamine for the growth and survival. The enzyme L-Glutaminase inhibits the proliferation as well causes the death of leukaemic cells by depriving them from L-Glutamine supply. The role of glutamine in prevention of chemotherapy and radiation induced toxicity is also evolving. The Glutamine supplement is inexpensive and is found to reduce the incidence of gastrointestinal, neurologic and possibly cardiac complications during career therapy.

A variety of microorganisms including bacteria, yeast, fungi have been reported to produce L-Glutaminase [1,6,7] of which, the most potent producers are fungi[8]. It has received a great significance since they are recognized as a anti-leukaemic agent and in food industries imparting the flavor and aroma to the foods, biosensors for monitoring L-Glutaminase levels for production of specific chemicals like theanine [9]. Marine microorganisms hold significance in food industries by virtue of their ability to produce salt tolerant L-Glutaminase. On industrial scale, Glutaminase production is mainly by *Aspergillus* and *Tricoderma sps* [10,11,12].

Different methods of fermentation technology can be applied for the production of Glutaminase. Commercially Glutaminase production has been carried out using SMF technique [13,14]. Now a days SSF has been gained a eminent role for the development of industrial bioprocess, especially due to low energy requirement and high product yields, less waste water production with less risk of bacterial contamination. In addition, it is eco friendly. The primary advantage of SSF is that many metabolites are produced at higher concentration. There are a few reports on the production of extracellular L-Glutaminase under SSF using microbial strains [1,15,16].

In the present study, the potential of *Aspergillus Wentii* MTCC 1901, for the production and optimization of extracellular L-Glutaminase using mixed substrate (Manila Tamarind, Palmyra Tuber and Tapioca) by SSF was evaluated.

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II. MATERIALS AND METHODS

A. *Substrate Collection*

Three different substrates namely Manila Tamarind (*Pithecellobium dulce*), Palmyra Tuber (*Borassus flabellifer*) and Tapioca (*Manihot esculenta*) were collected from local market of Visakhapatnam, India. The substrates were sun dried and ground to fine powder and used for the production of L-Glutaminase by *Aspergillus Wentii.*

B. *Microorganism Collection*

Pure culture of *A.wentii* MTCC 1901 was obtained from Institute of Microbial Technology, Chandigarh, India for the fermentation study.

C. *Growth medium and Culture Condition*

The culture was maintained on PDA slants having the composition (g/L): Potatoes infusion from 200, Dextrose 20 and agar-agar 15 with pH 5.6. 39 gm of PDA medium and 0.1 gm agar-agar were taken in 1000ml distilled water and used as growth medium. The culture was incubated at 28^0C for 120h. Sub culturing was carried out once in every 15 days and the culture was stored at 4^0C.

D. *Inoculum Preparation*

The spore suspension was prepared by scrapping on the surface of freshly raised 6 day old culture of fungi, *Aspergillus wentii* using Tween 80 solution.

E. *Fermentation Medium And Culture Conditions*

Solid state fermentation was carried out as indicated by El. Sayad [18] . 5gm of each substrate was taken in a 250ml Erlenmeyer flasks separately and moistened with 2 ml of distilled water. The flasks were autoclaved at 121^0C (15lb) for 20 min, cooled to room temperature and then inoculated with 2ml of *A.wentii* spore suspension under aseptic conditions. Then inoculated flasks were mixed thoroughly and incubate at 30^0C for 144hrs.

F. *Mixed Subtrate Composition*

Out of three substrates, two potential substrates Manila Tamarind powder and Palmyra Tuber powder were screened out. Substrates are mixed in different composition (0:5, 5:0, 1.5:3.5, 3.5:1.5 and 2.5:2.5). High yield was obtained with ratio of 3.5:1.5 (Manila Taramind : Palmyra tuber) and proved the best composition.

G. *Crude Enzyme Extraction*

After incubation period, the crude enzyme was extracted by using 0.1M phosphate buffer (pH 6.8). 41 ml of phosphate buffer was added to the fermented substrate and mixed well and the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was filtered by waltman filter paper and the filtrate was centrifuged at 10,000 rpm for about 10 min at 4^0C in a cooling centrifuge. The collected supernatant was used for enzyme assay [18].

H. *L-Glutaminase Assay*

The activity of L-Glutaminase was determined by estimating the amount of Ammonia liberated from L-glutamine by following the method of Imada *et al.* [13], which was given below. The enzymatic reaction mixture contains 0.5ml of L-glutamine (0.04M), 0.5ml of Tris-HCl buffer 0.1M (pH 6.8), 0.5ml of enzyme solution and distilled water to a total volume of 2.0ml was incubated at 37^0C for 30 min. The reaction was stopped by adding 0.5ml of 1.5 M Trichloro-acetic acid (TCA). Then to 3.7ml distilled water, 0.1 ml of the above mixture and 0.2ml of Nessler's reagent was added and colour developed was read after 10-15 min at 450nm in a UV-Visible spectrophotometer. The ammonium concentration of the reaction was determined by the reference from the standard curve of ammonium sulphate. One unit (U) of L-Glutaminase activity was defined as the amount of enzyme that liberates 1µmole of ammonia under optimal assay conditions. Enzyme yield was expressed as the activity of L-Glutaminase per gram dry substrate (U/gds).

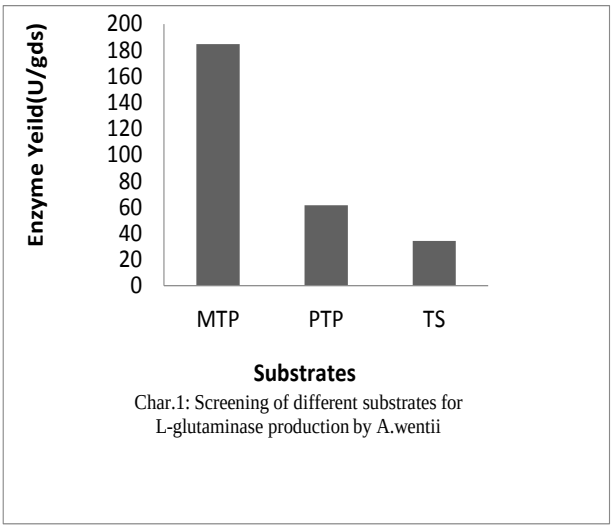
I. *Optimasation Of Process Parameters For L-Glutaminase Production*

The impact of various process parameters influencing L-Glutaminase synthesis by *A.Wentii* under Solid state Fermentation was studied. The effect of process parameters on enzyme production was determined by incubating with different composition of substrates, pH (3.0-9.0) adjusted with 1N HCL or 1N NaOH, temperature (25^0C-55^0C), moisture content (25-75%), incubation period (3-8days), addition carbon sources, nitrogen sources, metal ions and glutamine concentration at 1%W/V.

III. RESULT AND DISCUSSION

A. *Substrate Selection*

In the solid state fermentation the substrates used are in solid state. The filamentous fungi have the capacity to deeply penetrate the solid particles for up taking the nutrients. In the present investigations powder of Borassus flabellifer (Palmyra Tuber)(PTP), Pithecellobium dulce (Manila Tamarind)(MTP) and Manihot esculenta (Tapioca Starch)(TS) substrates are utilised for the growth of the fungus. In the reports, it observed that MTP obtained maximum yield (184.5 U/gds).



Char.1: Screening of different substrates for L-glutaminase production by A.wentii

B. *Mixed Substrate Composition*

The mixed substrate composition suitable for maximum Glutaminase yield was observed with 4 combinations of Palmyra tuber powder and Manila Tamarind powders. From

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the results it is observed at the ratio of 3.5:1.5 of Manila Tamarind powder and Palmyra tuber powder respectively has yield 280.166 U/gds was observed. Hence the mixed substrate ratio contains 3.5grams Tamarind and 1.5grams Palmyra has taken throughout the research work.

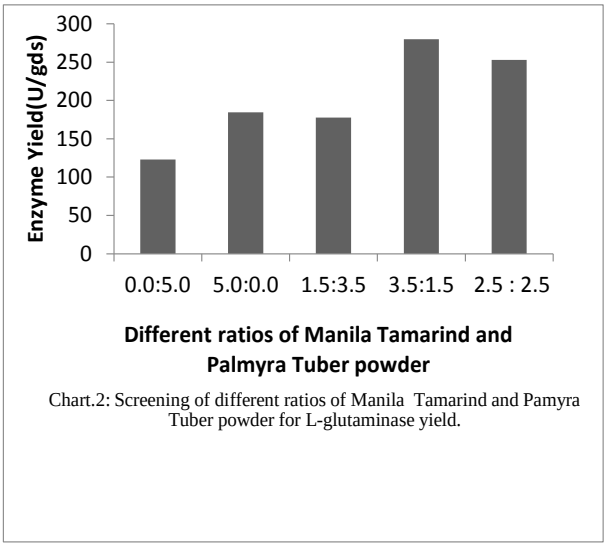

Chart.2: Screening of different ratios of Manila Tamarind and Pamyra Tuber powder for L-glutaminase yield.

C. *Effect Of Incubation Time*

In the present study the SSF was carried out for a period of 144 hrs. The enzyme assay was carried out for every 24hrs. The maximum enzyme production (177.66 U/gds) was observed on 6th day of incubation (fig.1). The further incubation after 6 days results in the lower enzyme yield. Hence the optimum incubation time was observed as 6 days. Most of the fungal strains produced maximum enzyme on 5th to 7th day of incubation [12,20].

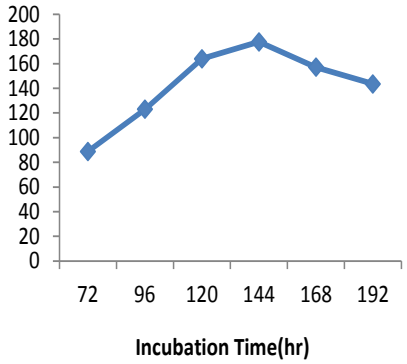

Fig.1: Effect of Incubation Time on L-Glutaminase Production.

D. *Effect Of Moisture Content*

Different levels of Moisture content was investigated during fermentation process by adjusting various moisture content levels are 25, 35, 45, 55, 65, 75 (%v/w) of substrate using distilled water. Addition of 1.25 to 3.75ml distilled water was taken in to account to calculate the moisture content and to study their effect on L-Glutaminase production. The maximum yield (184 U/gds) of L-Glutaminase enzyme was observed at 45% (v/w) initial moisture content (fig.2). Optimization of moisture content is the important parameter to regulate and to modify metabolic activity of microorganism [23,24,25].

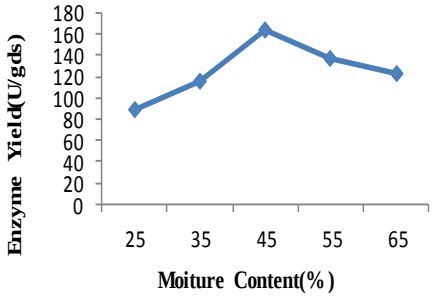

Fig.2: Effect of Moiture Content on L-Glutaminase Production.

E. *Effect of Temperature*

The solid state fermentation (SSF) was carried out at different temperatures ranging from 20^0C to 55^0C. The maximum Glutaminase yield was observed at 30^0C (350.15 U/gds) as shown in the fig.3.

The 30^0C was observed as optimum temperature for the maximum enzyme production. The significance of incubation temperature in the development of a biological process, which promotes the maximum enzyme production by the fungus. Similar observations were reported for Glutaminase from *Tricoderma koningii* [20] and *Aspergillus flavus* [21].

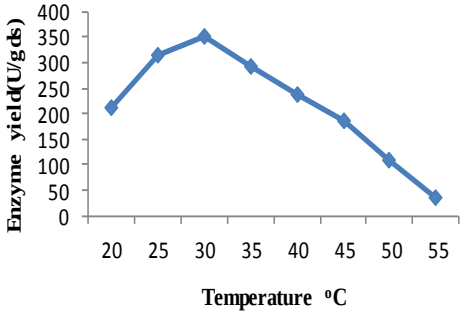

Fig.3: Effect of Temperature on L-Glutaminase Production

F. *Effect of pH*

The pH of the medium influence the growth and activity of the fungus. The pH of the buffer was taken from 5 to 9 and the maximum yield of enzyme observed at pH 6.8 (300.66 U/gds) fig.4. The results obtained are in agreement with *T. Konignii* was favourable for high enzyme yield [12,20]. Generally the fungal strain produces enzymes in the pH range of 3.0-7.0. The low pH values of the production media favours to avoid the contamination by other microorganisms [22].

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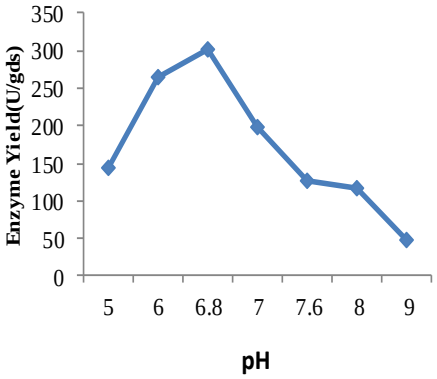

Fig.4: Effect of pH on L-Glutaminase Production.

G. Effect of Inoculum

The effect of inoculum volume plays a major role in Glutaminase production. Various volumes of inoculum concentrations (0.5 - 2.0 ml) were taken in different flasks during fermentation process. Out of these fermented flasks, the maximum yield (375.83 U/gds) was reported by flask containing 2ml volume of inoculum concentration of 6 day old fungal culture. But from other flasks low Glutaminase yield was observed.

In the fermentation process, levels of inoculum volume are very important to show optimal inoculum level. High level of inoculum cause more enough biomass leads to deplete the substrate of nutrients or accumulation of some non volatile self inhibiting the substance inhibiting the product formation. Whereas low inoculum levels may leads to insufficient biomass and reduced product formation [1].

In this fermentation process, the optimum inoculum volume (2ml) balances the proliferating biomass and availability of nutrient that supports maximum enzyme production (fig.5).

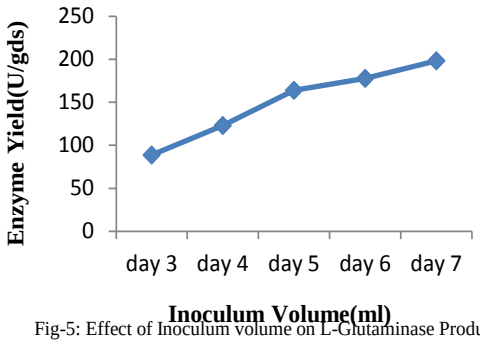

Fig-5: Effect of Inoculum volume on L-Glutaminase Production.

H. Effect Of Carbon Sources

In the Glutaminase production, incorporation of additional carbon sources to the basal solid fermentation medium of *A.Wentti* extracted a considerable effect on biosynthesis of L-Glutaminase production. The carbon sources like D-Glucose, Maltose, Fructose, Lactose and Sucrose at 1% (W/V) were used. Compared to other carbon source, a stimulatory effect was observed in Glucose (410 U/gds) followed by Maltose (266.5 U/gds) stimulatory influence of Maltose act as a cometabolic agent (Fig-6).

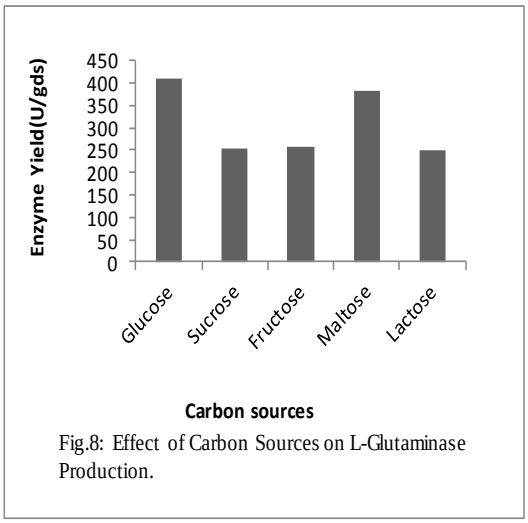

Fig.8: Effect of Carbon Sources on L-Glutaminase Production.

I. Effect of Nitrogen Sources

Nitrogen sources are important enhancing or limiting factor for microbial growth of production. The different nitrogen sources (Peptone, Ammonium nitrate, Ammonium sulphate, sodium nitrate, Yeast extract) of 1% (w/v) are tested. Among peptone reported as a source of maximum yield of enzyme (471.5 U/gds)(Fig.7). Later ammonium nitrate showed the yield of 457.8 U/gds. The least effect was observed in Ammonium sulphate (334.8 U/gds).

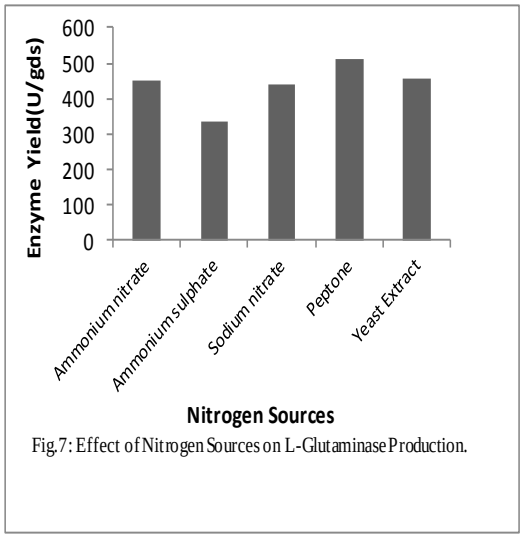

Fig.7: Effect of Nitrogen Sources on L-Glutaminase Production.

J. Effect Of Metal Ions

Different metal ions like Magnesium Sulphate, Sodium Sulphate, Calcium Chloride, Potassium Chloride and Zinc Sulphate were added to enhance the production. All metal ions showed a slightly increase in enzyme production. The maximum yield was reported by Sodium Chloride (594.5U/gds) (Fig-8).

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This suggests that the metal ions are useful for the improvement of Glutaminase production as the metals might act as co-factor for many enzymes involved in intermediatory metabolism.

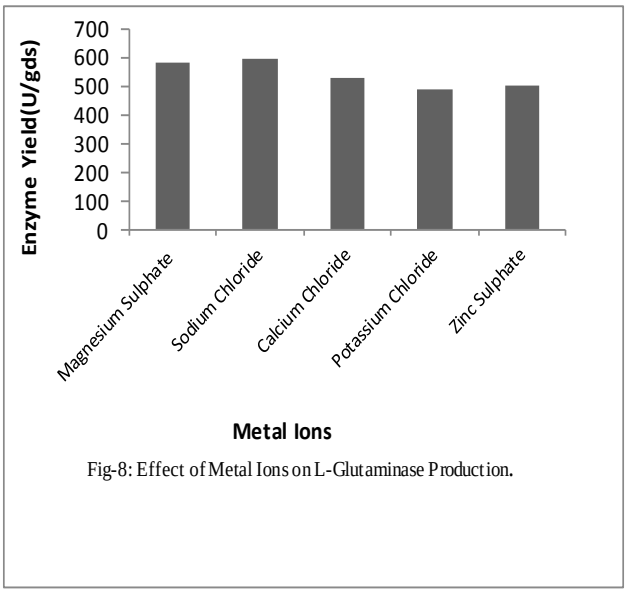

Fig-8: Effect of Metal Ions on L-Glutaminase Production.

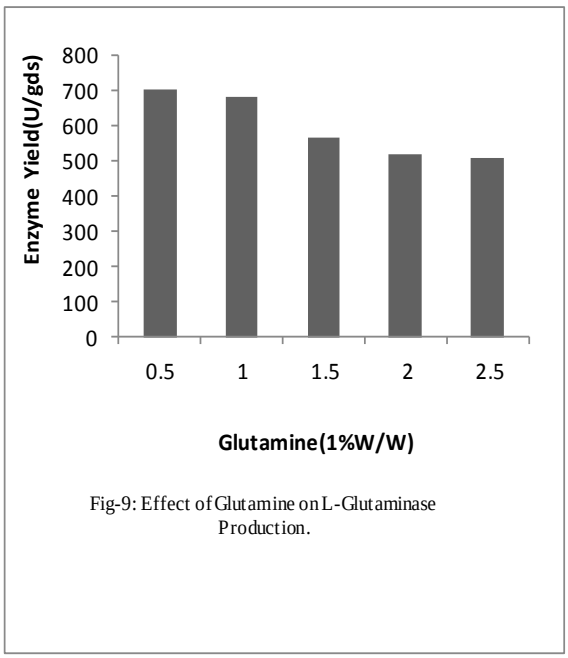

Fig-9: Effect of Glutamine on L-Glutaminase Production.

K. Effect Of Glutamine

Glutamine increase stability and stimulates Glutaminase production. Addition of glutamine to fermentation medium enhances the enzyme production. Glutamine concentration used for the substrate at an optimum of 1% (W/W). Though maximum yield (703.8U/gds) was reported at the concentration of 0.5% (Fig.9).

IV. CONCLUSION

This study has been carried out to explore the possibility of using mixed substrates of agro residues Manila Tamarind Powder and Palmyra Tuber powder for the production of L-Glutaminase using Solid state Fermentation. The fungal strain *Aspergillus Wentii* MTCC 1901 has produced highly effective L-Glutaminase by using mixed substrates of agro residues by SSF. The optimization of fermentation processes resulted in the 3.8 fold increased in the production of effective L-Glutaminase (703.83 U/gds). It proved as a prospective technique for the large scale production of the L-Glutaminase, which is gaining vast significance in pharmaceutical and food industries.

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