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L-GLUTAMINASE PRODUCTION BY *ASPERGILLUS WENTII* MTCC 1901 UNDER SOLID STATE FERMENTATION USING MIXED AGRO INDUSTRIAL RESIDUES

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ABSTRACT

Optimization of physicochemical parameters for production of extracellular L-glutaminase using *Aspergillus wentii* MTCC 1901 under solid state fermentation is detailed. Substrate screening showed significant results for wheat bran, sesame oil cake (SOC) and green gram husk (GGH). Furthermore, 1 : 1 combination of SOC and GGH showed best results among six different mixed substrate combinations performed. The maximum activity (259.32 U/gd s) of L-glutaminase by *Aspergillus wentii* MTCC 1901 was obtained using SOC : GGH (1 : 1) of 40% initial moisture content, initial pH 8.0, supplemented with sucrose (4.0% w/v), peptone (2.0% w/v), magnesium sulphate (1% w/v) and L-glutamine (3.0% w/v), inoculated with 2 mL of 96 hours old fungal culture and incubated at 32^{o}C for 96 hours. Both physical and chemical parameters had played a significant role in the production of L-glutaminase. Medium with optimized conditions and supplementation gave a maximum glutaminase activity of 259.32 U/gds, which is above 6 fold increase in activity than that with medium of basal conditions (39.9 U/gds). An anti-leukemic agent L-glutaminase with relatively good activity is produced from agro industrial residues in a mixed substrate combination.

Key words: L-Glutaminase, *Aspergillus wentii*, Solid state fermentation, Optimization.

INTRODUCTION

L-Glutamine amido hydrolase (EC 3.5.1.2) commonly referred to as L-glutaminase is a hydrolytic enzyme that deaminates glutamine to glutamate and ammonia. This action of L-glutaminase has a pivotal role in cellular nitrogen metabolism[1-3]. In recent years, microbial L-glutaminase has gained prime importance in food and pharmaceutical industries as an effective agent in the treatment of acute lymphocytic leukemia[4] and HIV[5,6], as an analytical

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agent[7,8], as a bio-sensing agent[9], as a flavour enhancing agent[10], and in the production of speciality chemicals like threonine by gamma glutamyl transfer reactions[11].

Although almost all living cells produce L-glutaminase, microbial L-glutaminases has received the greater attention because of its apparent advantages in production at large scale in addition to its applications. On an industrial scale, glutaminases are produced mainly by *Aspergillus* and *trychoderma* sp[12]. Commercial production of glutaminases has been carried out using submerged fermentation (SMF) technique[13]. But nowadays, solid state fermentation (SSF) has been emerging as a promising technology for the development of several bioprocesses and products including the production of therapeutic enzymes on a large scale. The product titers produced in SSF are many folds higher than submerged culture, although the reasons for this are not clear[14]. SSF also appears to possess several biotechnological advantages such as higher fermentation productivity, higher product stability, lower catabolic repression and lesser demand on sterility owing to the low water activity used in SSF[15].

To our knowledge most of the reports deal with single solid substrate rather than mixed composite in the production of L-glutaminase; however, fewer reports are available on mixed substrate design for solid state fermentation. In the present paper, mixed agro industrial residues were chosen for the production of L-glutaminase by *Aspergillus wentii* MTCC 1901 under solid state fermentation.

EXPERIMENTAL

Microorganism and inoculum preparation

The fungal strain *Aspergillus wentii* MTCC 1901 used in this study was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on potato dextrose agar (PDA) medium. The microbial strain was grown at 28^{o}C for 5 days after which, it was stored at 4^{o}C until further use. The organism was sub-cultured for every 4 weeks. Spore suspension was prepared from a freshly raised 5 day old culture of *A. wentii* MTCC 1901 on PDA slants by suspending in 10 mL of 0.1% Tween 80 solution.

Fermentation medium and culture conditions

Fermentation was carried out under solid state fermentation as done by Ashraf et al.[16] The fermentation medium has the following composition in a 250 mL Erlenmeyer flask: 5 g of mixed substrates were taken separately and moistened with 2 mL of moistening medium (distilled water). The flasks were autoclaved at 121^{o}C (15 lb.) for 20 min, cooled to room

temperature and then inoculated with 2 mL of the *A. wentii* MTCC 1901 spore suspension under aseptic conditions. The contents of the inoculated flasks were mixed thoroughly and incubated at the desired temperature in an incubator for desired length of period.

Optimization of the culture condition for L-glutaminase production

Various process parameters that enhance the activity of L-glutaminase by *A. wentii* MTCC 1901 under solid state fermentation were investigated. The impact of incubation period (0-216 h), incubation temperature (26-40^{o}C), initial moisture content of the substrate (20-90%), initial pH (5-10) adjusted with 0.1 N HCl or 0.1 N NaOH, inoculum age (48-192 h) and inoculum volume (1-4 mL), were evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, maltose, sucrose, fructose, lactose, galactose, mannose and soluble starch at 1% w/v); nitrogen sources (sodium nitrate, ammonium sulphate, malt extract, yeast extract, urea and peptone at 1% w/v) and metal salts (magnesium sulphate, calcium chloride, sodium chloride, potassium dihydrogen orthophosphate) were also studied. All the experiments were conducted in duplicate and the mean values were reported.

Crude enzyme extraction

After the incubation period, the crude enzyme from the fermented substrate was extracted using 0.1 M phosphate buffer (pH 8.0). After mixing the fermented substrate with 41 mL of buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was centrifuged at 10,000 rpm for about 10 min at 4^{o}C in a cooling centrifuge. Supernatant was collected and used for enzyme assay.

L-Glutaminase assay

The activity of L-glutaminase was determined by estimating the amount of ammonia liberated from L-glutamine using the method of Imada *et al.*[13] The enzymatic reaction mixture contains 0.5 mL of L-glutamine (0.04 M), 0.5 mL of tris-HCl buffer 0.1 M (pH 8.0), 0.5 mL of enzyme solution and distilled water to a total volume of 2.0 mL was incubated at 37^{o}C for 30 min. The reaction was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). Then to 3.7 mL distilled water, 0.1 mL of the above mixture and 0.2 mL of Nessler's reagent was added and colour developed was read after 10-15 min at 450 nm in a UV-spectrophotometer. The ammonia concentration of the reaction was determined by the reference from the standard curve of ammonium sulphate.

One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1 µmole of ammonia under optimal assay conditions. Enzyme activity was expressed as the activity of L-glutaminase per gram dry substrate (U/gds).

RESULTS AND DISCUSSION

The solid substrates used in solid state fermentation are generally insoluble in water and play a dual role of supply of nutrients to the microbial culture growing and anchorage for the growing cells. In SSF, the selection of a suitable substrate for a fermentation process is a critical factor and thus, involves the screening of a number of agro industrial residues for the microbial growth and product formation. In the present study, seven substrates, viz. rice bran, wheat bran, black gram husk, green gram husk, coconut oil cake, ground nut oil cake and sesame oil cake were initially screened with *Aspergillus wentii* MTCC 1901. The three substrates wheat bran, sesame oil cake, and green gram husk giving significant activity were mixed in 6 different combinations. The maximum L-glutaminase activity of 39.9 U/gds (Table 1) was achieved in a medium containing 1 : 1 combination of sesame oil cake and green gram husk.

++ Enzyme activity > 35; + Enzyme activity > 20; - Enzyme activity < 10

Table 1: Screening of different agro-industrial residues for L-glutaminase production by *A. wentii* MTCC 1901

S. No.	Wheat bran % [grams]	Green gram husk% [grams]	Sesame oil cake% [grams]	Enzyme activity U/gds
1	50 [2.5]	50 [2.5]	0 [0]	-
2	**0 [0]**	**50 [2.5]**	**50 [2.5]**	**++**
3	33 [1.65]	33 [1.65]	33 [1.65]	+
4	66 [3.3]	16.6 [0.83]	16.6 [0.83]	-
5	16.6 [0.83]	66 [3.3]	16.6 [0.83]	-
6	16.6 [0.83]	16.6 [0.83]	66 [3.3]	+

Optimization was done on “One parameter at a time basis” i.e., by changing one independent variable while fixing the others at a certain constant level. Optimum conditions obtained in each stage are applied to subsequent experiments.

From the above table, it is clear that the combination of sesame oil cake and green gram husk in 1 : 1 ratio showed highest activity.

Effect of incubation period

SSF was conducted for a period of 216 h at regular intervals of 24 h to evaluate the optimum time of fermentation for the enzyme production. The enzyme production showed growth relatedness as the incubation period progressed and maximum enzyme production (47.88 U/gds) was observed at 96 h (Fig. 1). Fermentation beyond 96 h showed a decrease in enzyme production, which could be either due to the inactivation of the enzyme because of the presence of some kind of proteolytic activity or the growth of the organism might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient resources. Nathiya et al.[17], reported 120 hours of incubation period using *Aspergillus fumigates* in solid state fermentation, which is in near agreement with the current readings.

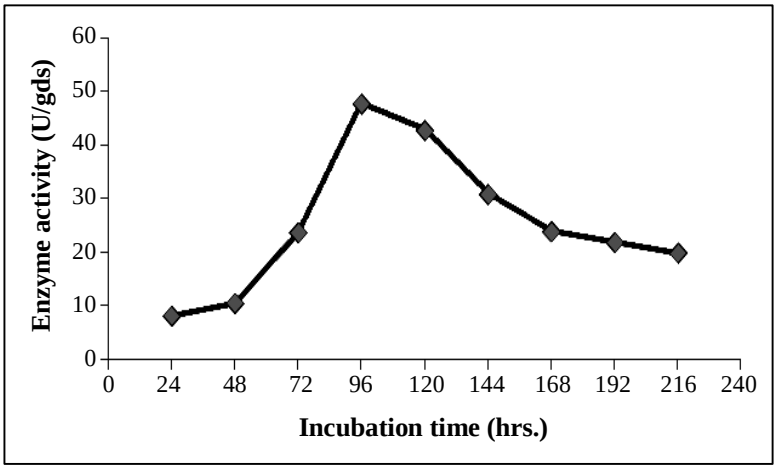

Fig. 1: Effect of incubation period on L-glutaminase production

Effect of temperature

Fermentation was carried out at various temperatures such as 26, 28, 30, 32, 34, 36, 38 and 40^{o}C to study their effect on enzyme production. Incubation temperature has shown considerable effect on L-glutaminase production by *A. wentii* MTCC 1901 under solid cultural conditions. The maximum enzyme activity of 61.84 U/gds was obtained when SSF was carried out at 32^{o}C (Fig 2). However, the enzyme activity reduced gradually with further increase in incubation temperature above 32^{o}C. This may be due to the denaturation of microbial strain at higher temperatures. These results were in coincidence with those reported by Chanakya et al.[18]

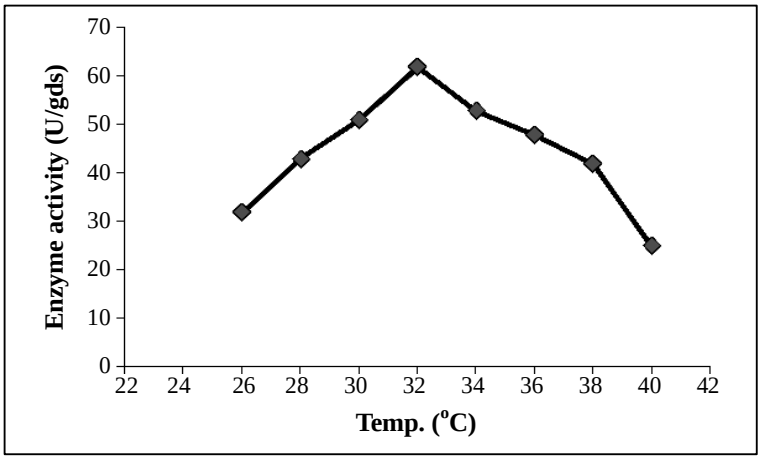

Fig. 2: Effect of temperature on L-glutaminase production

Effect of initial moisture content

To investigate the influence of initial moisture content of the substrate, fermentation was carried out with various initial moisture content levels viz. 20, 30, 40, 50, 60, 70, 80 and 90 (% v/w) of 1 : 1 SOC and GGH, adjusted with distilled water. Maximum enzyme production of 70.22 U/gds was achieved at 40% initial moisture content (Fig. 3). A further increase in the initial moisture content beyond 40% resulted in a significant reduction in the enzyme production. The inhibitory effect on enzyme production at higher moisture content may be due to substrate particle agglomeration, lower O_2 transfer, decrease in porosity and enhancement of bacterial growth. Similar results of the effect of moisture content on L-glutaminase production by *Aspergillus flavus* was reported by Nathiya et al.[17]

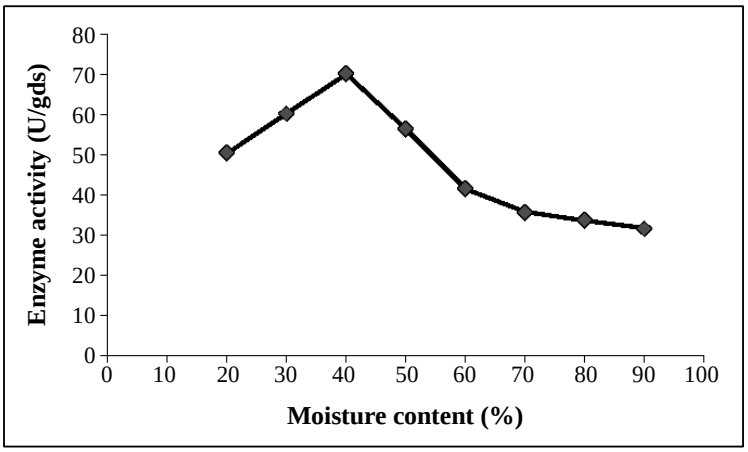

Fig 3: Effect of initial moisture content on L-glutaminase production

Effect of inoculum age

Fermentation was carried out with different inoculum ages ranging from 24-192 h for a period of 24 h to study its effect on L-glutaminase production. Maximum enzyme production (87.78 U/gds) was obtained with 96 h old culture of *A. wentii* MTCC 1901 (Fig 4). Further increase in the inoculum age resulted in the decrease of enzyme production, which may be due to the occurrence of microbial death phase.

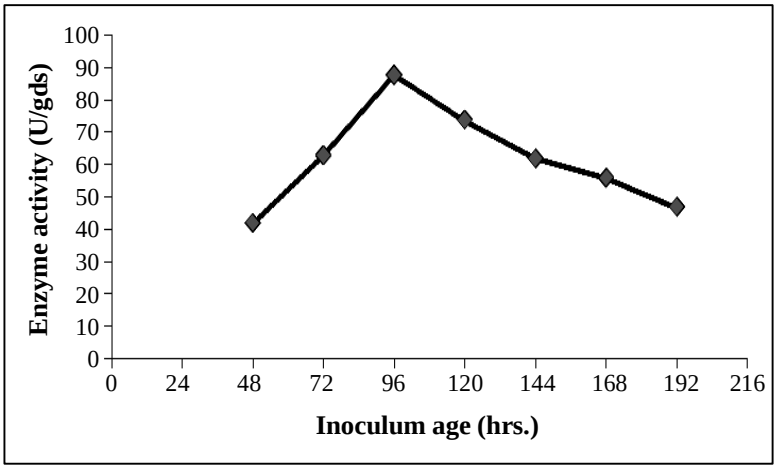

Fig 4: Effect of inoculum age on L-glutaminase production

Effect of inoculum volume

Fermentation was carried out with different inoculum volumes viz 1, 1.5, 2.0, 2.5, 3.0, 3.5 and 4 mL for a period of 96 h to study its effect on the production of L-glutaminase. Maximum L-glutaminase production of 99.70 U/gds was obtained with 2 mL of 4 day old culture of *A. wentii* MTCC 1901 (Fig 5). With further increase in inoculum volume; there was a gradual decrease in the enzyme production and microbial activity, which might be attributed to the nutrient limitations[8].

Effect of initial pH

In order to maintain the favourable conditions for increased L-glutaminase production, pH was optimized. This was established by carrying out the fermentation by varying the pH from 4-10 (adjusted with 0.1 N HCl or 0.1 N NaOH). The significance of initial pH of the fermentation on the production of L-glutaminase production was observed. The maximum L-glutaminase production of 35.39 (U/gds) was obtained at pH 8.0 (Fig 6).

Similar results of the effect of pH on L-glutaminase production using *Zygosaccharomyces rouxii* was reported by Iyer and Singhal[19].

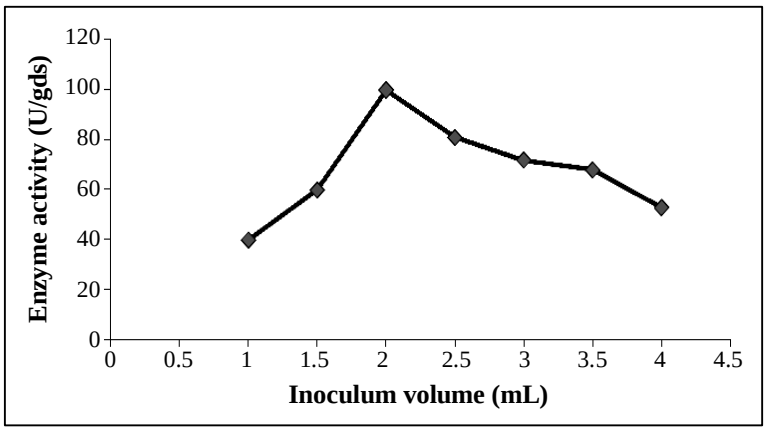

Fig. 5: Effect of inoculum volume on L-glutaminase production

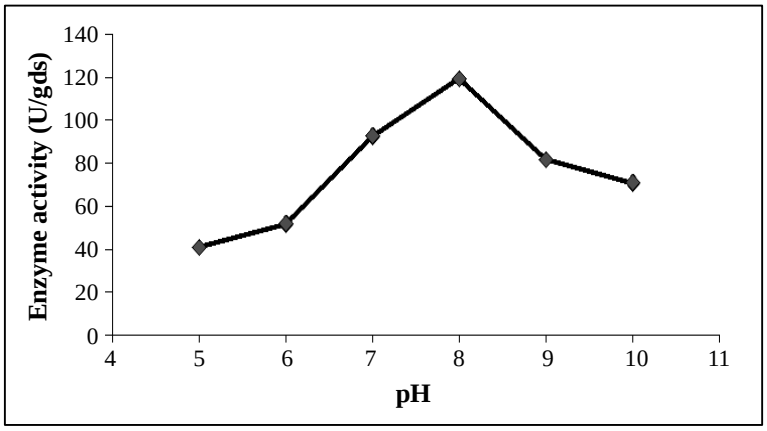

Fig 6: Effect of initial pH on L-glutaminase production

Effect of carbon source

The incorporation of additional carbon sources might enhance the enzyme production. So, different carbon sources namely glucose, maltose, starch, sucrose, fructose, maltose, galactose, lactose and mannose at 1% (w/v) were added to the basal solid state fermentative medium containing *A. wentii* MTCC 1901 and they have exerted a considerable effect on the biosynthesis of L-glutaminase. The maximum enzyme production was promoted by sucrose followed by glucose and soluble starch (Fig 7). Similar results were reported by production by *Trichoderma koningii* by Ashraf *et al.*[16].

Effect of Sucrose concentration

In order to investigate the effect of sucrose concentration on the fermentation medium, SSF was carried out with different sucrose concentrations varying from 1-6% (w/v). From the data obtained, it can be concluded that the maximum production of L-glutaminase (158.51 U/gds) was obtained with the optimum sucrose concentration of 4% (w/v) (Fig 8). Further increase in sucrose concentration resulted in the decrease of enzyme production. Similar results of the effect of sucrose and glucose concentration on L-glutaminase production was reported by Sabu et al.[9]

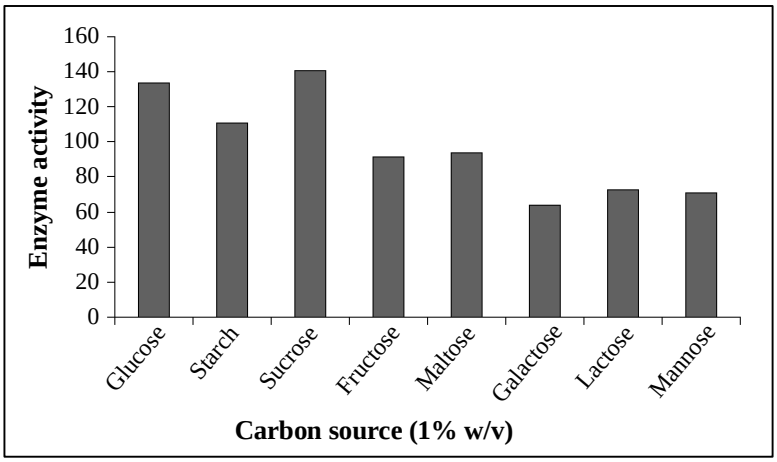

Fig 7: Effect of carbon source on L-glutaminase production

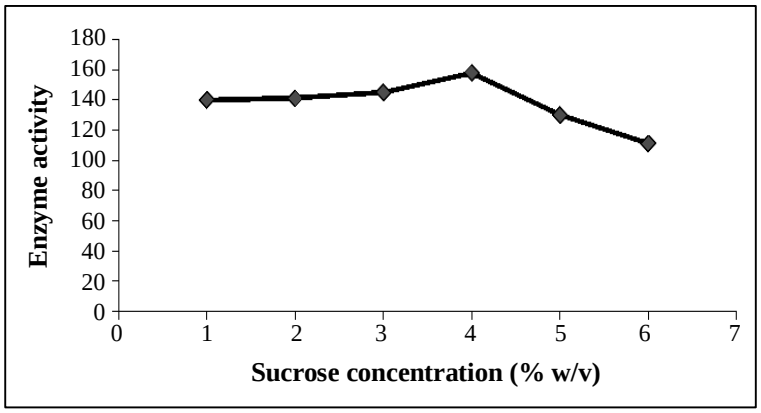

Fig 8: Effect of sucrose concentration on L-glutaminase production

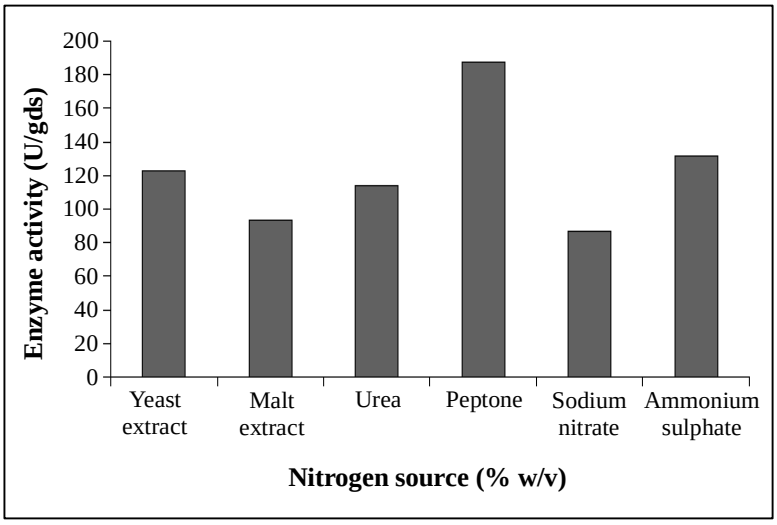

Fig 9: Effect of nitrogen source on L-glutaminase production

Effect of nitrogen source

Nitrogen source can be an important limiting factor in the microbial production of enzymes[4]. The supplementation of additional nitrogen sources (either organic or inorganic) such as ammonium sulphate, sodium nitrate, malt extract, yeast extract, urea and peptone had shown a considerable impact on the production of L-glutaminase by *A. wentii* MTCC 1901 under SSF. Among the various nitrogen sources, peptone in the medium promoted enhanced growth of microorganism and consequently the L-glutaminase production, followed by ammonium sulphate and yeast extract (Fig 10). Similar results of the effect of nitrogen sources on L-glutaminase production in solid state fermentation using *Aspergillus flavus* was reported by Nathiya *et al.*[17]

Effect of peptone concentration

In order to evaluate the effect of peptone concentration on the fermentation medium, SSF was carried out with different concentrations of peptone varying from 1-6% (w/v). The results revealed that the maximum L-glutaminase production (219.40 U/gds) was obtained with peptone of 2% (w/v). Further increase in peptone concentration resulted in the decrease of enzyme production due to the repressor effect of peptone at higher concentrations. Similar results of the effect of nitrogen sources like yeast extract and peptone on L-glutaminase production were reported by Nathiya *et al.*[17]

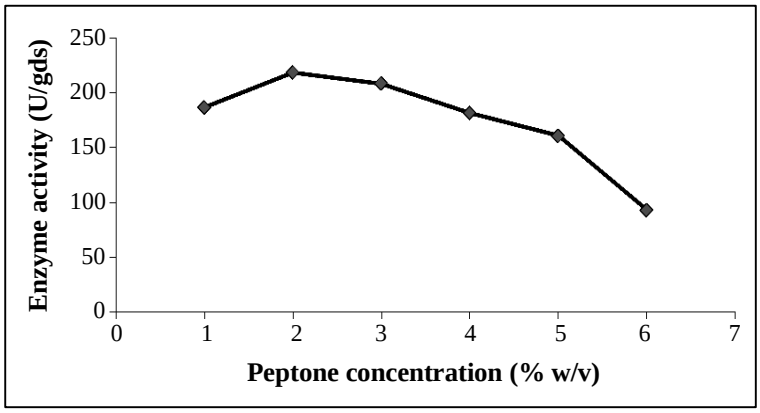

Fig 10: Effect of peptone concentration on L-glutaminase production

Effect of metal salts

The effect of metal salts on L-glutaminase production was determined by adding different metal salts to the fermentation medium. Metal salts provide metal ions that are essential for cell mass formation and also act as cofactor for several biosynthetic enzymes. The metal salts selected were magnesium sulphate, calcium chloride, sodium chloride and monobasic potassium phosphate at 0.1% (w/v) concentration. Addition of magnesium sulphate to the fermentation medium showed high L-glutaminase activity of 163.34 U/gds. Nathiya *et al.*[17] reported that maximum activity was observed with $MgSO_4$ $7H_2O$ by *Aspergillus flavus* using tea dust as substrate in solid state fermentation.

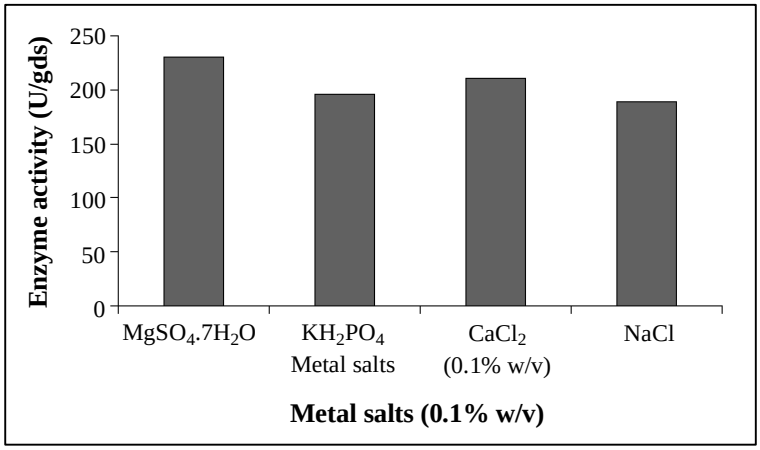

Fig. 11: Effect of metal salts on L-glutaminase production

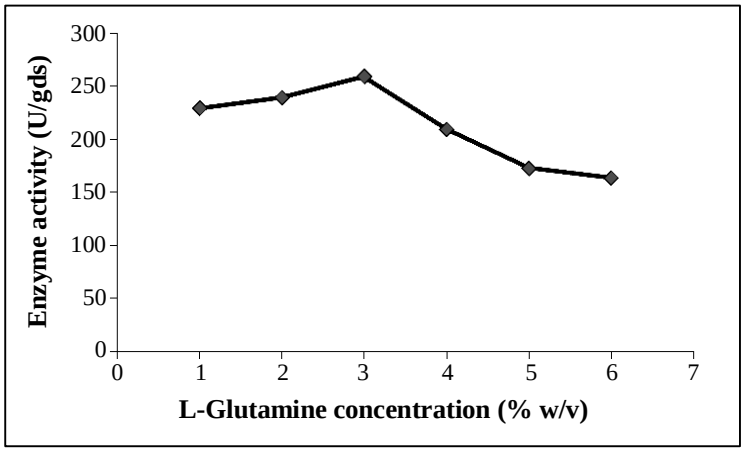

Fig. 12: Effect of L-glutamine concentration on L-glutaminase production

Effect of L-glutamine concentration

Since L-glutamine is the substrate of glutaminase, the addition to fermentation medium might stimulate enzyme production. So, to study its effect on L-glutaminase production, fermentation was carried out with different concentrations of L-glutamine ranging from 1-6 (% w/v) for a period of 96 h. The maximum enzyme production (259.32 U/gds) was observed with 3% (w/v). With further increase in L-glutamine concentration, there was a gradual decrease in enzyme production, which may be due to the inhibitory effect of L-glutamine at higher concentrations on the enzyme production. Similar results of glutamine supplementation in L-glutaminase production was reported by Kumar *et al.*[20]

Finally with all the optimized parameters, incubation time 96 h, incubation temperature 32^{o}C, initial moisture content 40 %(v/w), inoculum age 96 h, inoculum volume 2 mL, pH 8.0, sucrose 4% (w/v), peptone 2% (w/v), magnesium sulphate 1% (w/v), L-glutamine concentration 2% w/v, L-glutaminase activity of 259.32 U/gds was obtained.

Solid state fermentation revealed the possibilities of effective utilization of agro-industrial residues such as sesame oil cake and green gram husk for value addition through biotechnological means. The present study shows that *Aspergillus wentii* MTCC 1901 can produce an anti-leukemic agent L-glutaminase with relatively good activity from agro industrial residues in a mixed substrate combination of 1 : 1 sesame oil cake and green gram husk, which are easily available and economical. The significant improvement in L-glutaminase activity was recorded, when the basal medium was supplemented with different

carbon and nitrogen sources. Addition of metal salts also showed considerable effect on the activity enhancement. It is also observed that the addition of L-glutamine to the nutrient media resulted in the significant improvement of the activity of L-glutaminase. This process can be exploited for the large scale production of L-glutaminase, which has wider applications in the field of food and pharmaceutical industries.

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