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Full Length Research Paper

Optimised production of L-glutaminase: A tumour inhibitor from *Aspergillus flavus* cultured on agroindustrial residues

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L-Glutaminase, an amidohydrolase enzyme has been a choice of interest in the treatment of lymphoblastic leukemia. This study investigates the production and optimization of extracellular glutaminase enzyme using several agro-industrial residues by *Aspergillus flavus KUGF009* using SSF (solid state fermentation). Effect of process variables namely substrates, incubation period, temperature, moisture content, initial pH, supplementary carbon and nitrogen sources and metal ions on the production of L-glutaminase was studied and accordingly, optimum conditions were determined. *A. flavus* KUGF009 was cultured in tea dust to produce L-glutaminase. The organism produced high levels of glutaminase under optimized culture conditions on the 5th day of incubation at an optimum pH 4.0, temperature 30°C and moisture content 50% by SSF. Enhanced production occurred on the addition of dextrose, yeast extract and $MgSO_4$ as nutritional factors.

Key words: L-Glutaminase, anti-leukemic agent, *Aspergillus flavus*, solid state fermentation.

INTRODUCTION

L-Glutaminase is a potent anti-leukemic agent. Unlike normal cells, leukemic cell do not demonstrate the L-glutamine synthetase, thus, it depends on the exogenous supply of L-glutamine for their growth and survival (Rohde et al., 1996). Tumour cells have an absolute requirement for glutamine as a growth substrate. Glutamine is required as a precursor for both DNA synthesis and protein synthesis, and may also be used as a respiratory substrate. In experiments where glutamine metabolism in tumour cells has been specifically compared with that in non-transformed cells of the same origin, glutamine metabolism in the tumour cells has been found to be considerably faster (Souba, 1993).

A variety of microorganisms, including bacteria, yeast, moulds and filamentous fungi have been reported to produce L-glutaminase (Kashyap et al., 2002; Weingand-Ziade et al., 2003; Iyer and Singhal, 2008) of which the most potent producers are fungi (Balagurunathan et al., 2010). On an industrial scale, glutaminases are produced mainly by *Aspergillus* and *Trichoderma* sp. (Tomita et al., 1988; Masuoa et al., 2004; El-Sayed, 2009; Pallem et al., 2010). The enzyme L-glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which generates L-glutamic acid and ammonia from L-glutamine. 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.

The action of glutaminase plays a major role in the cellular nitrogen metabolism of both, prokaryotes and eukaryotes (Kashyap et al., 2002; Klein et al., 2002). In recent years, L-glutaminase has received attention as a therapeutic agent against cancer and HIV (Roberts et al., 1970; Chandrasekaran et al., 1991; Kumar and Chandrasekaran, 2003), as a bio-sensing agent in monitoring glutamine levels (Kashyap et al., 2002; Zhao et al., 2004), for the production of specialty chemicals like theanine by c-glutamyl transfer reactions and as a flavour enhancer in the food industry (Sivakumar et al., 2006). The use of L- glutaminase as a flavour-enhancing agent in

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Chinese foods has replaced the use of monosodium glutamate which acts as an allergen for individuals (Rohde et al., 1996; Masuoa et al., 2004; Thongsanit et al., 2008; Jeon et al., 2009).

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 (Nathiya et al., 2011). The objective of this study was to select fungus with good ability to produce glutaminase and optimize media composition for maximizing the glutaminase production.

MATERIALS AND METHODS

Microorganism

40 fungal species used in this study were isolated from various substrates like soya sauce, fungal infected coconut (wet kernel) and spoiled bread (Palaniswamy et al., 2008). The fungal cultures were maintained on potato-dextrose agar, incubated at 30°C for 7 days and then stored at 4°C until use.

Enzyme production

Five grams of agro industrial residues 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 which was prepared previously. The inoculated flasks were mixed thoroughly and incubated at 30°C for 5 days. The crude L-glutaminase was extracted with 40 ml of the citrate phosphate buffer (pH 7.0), centrifuged at 4°C for 20 min at 10,000 rpm.

The fungi which shows high productivity was subsequently identified as *Aspergillus flavus*, designated as *KUGF009*, was selected to study the optimal conditions for L-glutaminase production in solid state fermentation.

Impact of process parameters on L-glutaminase production

The impact of various process parameters influencing L-glutaminase synthesis by *A. flavus KUGF009* under solid state fermentation was studied. The effect of process parameters on enzyme production was determined by incubating at different agro industrial residues (blackgram husk (BG), bengalgram husk (BH), corn cob (CC), cottonseed (CS), groundnut oilcake(GC), greengram husk (GH), gingelly oilcake, (GOC), lemon peel (LP), orange peel (OP), rice bran (RB), tea dust (TW) and wheat bran (WB), pH (3 to 11 adjusted with 1 N HCl or 1 N NaOH), temperature (25 to 50°C), moisture content (10 to 60%), incubation period (3 to 7 days), additional carbon sources (sucrose, glucose, lactose, galactose and dextrose at 1% w/v), nitrogen sources (peptone, yeast extract, urea, casein and albumin) and metal ions ($CaCl_2$, KCl, $MgSO_4$, NaCl and $ZnSO_4$).

Glutaminase assay

Assay of glutaminase was carried out by slight modification of Imada et al. (1973) using L-glutamine as substrate. L-glutamine (1 ml of 1%) in 0.01 M phosphate citrate buffer (pH 7.0) was made to react with 1 ml of glutaminase enzyme for 60 min at 37°C. The enzymatic activity was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. The reaction mixture was centrifuged at 5,000 rpm for 5 min to remove the precipitated protein. Then 0.1 ml of earlier mentioned 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 (Wade et al., 1971). One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal assay conditions.

Statistical analysis

The statistical software, SPSS version 10.05 was used to estimate the statistical parameters. Values are mean ± SD (n=3).

RESULTS AND DISCUSSION

Effect of substrates

The critical factor in SSF (solid state fermentation) is the choice of a suitable substrate for the fermentation process (Nathiya et al., 2011). The screening of various substrates for maximum L-glutaminase production clearly depicts the variation on the enzyme productivity for the various substrates employed (Figure 1). The maximum enzyme productivity was observed for tea dust (42.37±0.47 U/g) followed by bengalgram husk, groundnut oilcake, wheat bran and rice bran. Tea dust is a suitable supporter and carrier because of its porosity and its availability. Mitchell and Lonsane (1992) reported that the enzyme production is often simple, when agro-industrial by-products like wheat bran, rice bran or wheat straw are used as substrate. Because the moisture level is low, the volume of medium per unit weight of substrate is low. Hence, enzyme activity is usually very high (Deschamps and Huet, 1885).

Effect of pH

The pH of the medium is one of the most critical environmental parameter affecting the mycelial growth, enzyme production and the transport of various components across the cell membrane (Kapoor et al., 2008). The results illustrated in Figure 2 showed that the initial pH of the medium (El-Sayed, 2009) was suitable to enhance growth of *A. flavus KUGF009* to produce high quantity of the enzyme (38.69 ± 0.29 U/g). Increasing the initial pH of the medium from 4 to 7 showed a significant decrease in the production of L-glutaminase enzyme. A loss of more than 50% in enzyme production was observed at medium pH of 7. Our results obtained are in agreement with Sivakumar et al. (2006) who produced productivity of 14.74 IU/ ml. Fungal strains are noted for their best performance in the range of 3.5 to 7.0 and also low pH avoids the contamination by other microbes (Pandey et al., 2001).

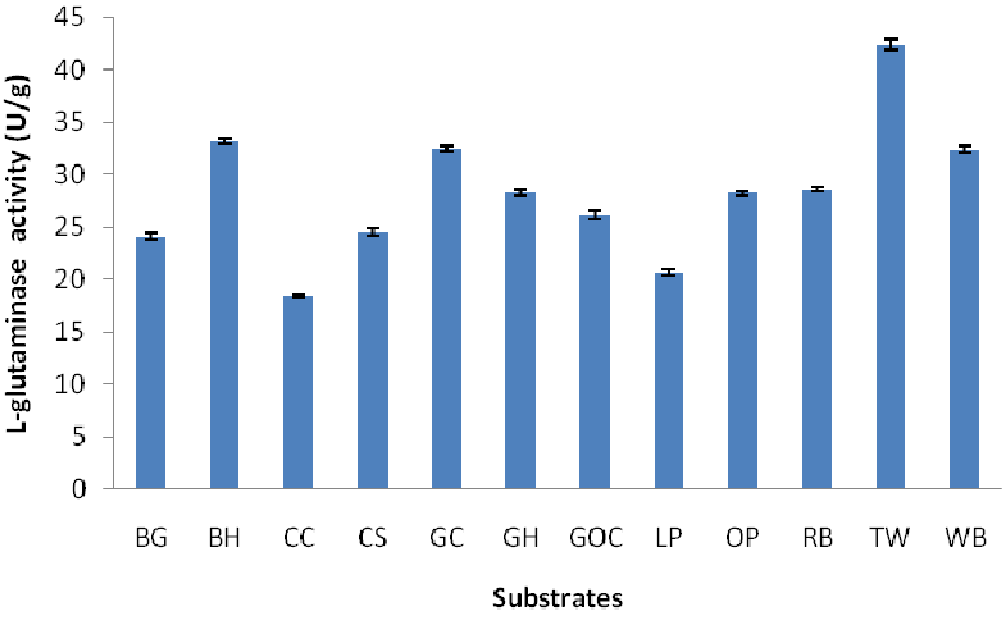

Figure 1. Effect of substrates on L-glutaminase production.

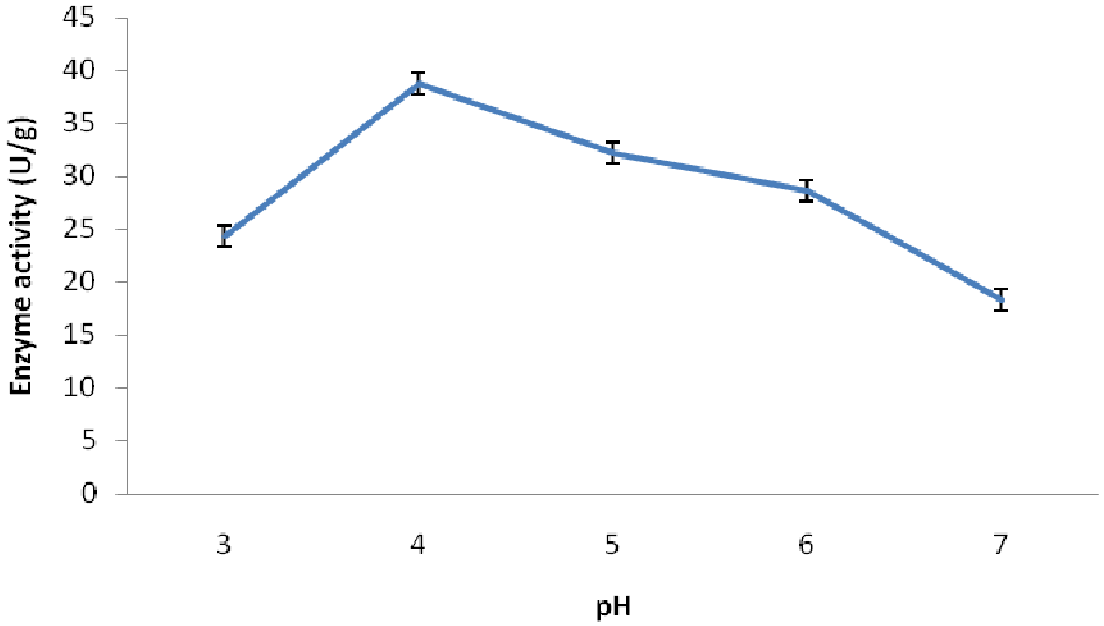

Figure 2. Effect of pH on L-glutaminase production.

Effect of temperature

Among the different temperatures tried, the maximum enzyme production was observed at 30°C (58.29 ± 0.33 U/g). The optimum temperature for the enzyme activity was found to be 30 to 40°C, at which the enzyme activity was the highest (Figure 3). Although, the physiological changes induced by high temperatures during enzyme production are not completely understood, it has been suggested that at high temperatures, microorganisms may synthesize only a reduced number of proteins essential for growth and other physiological processes (Gawande and Kamat, 1999). Similar observations were reported for glutaminase from *Trichoderma koningii* which produced 15.59 U/gds at 33°C (El-Sayed, 2009). With prolonged incubation, enzyme activity decreased sharply

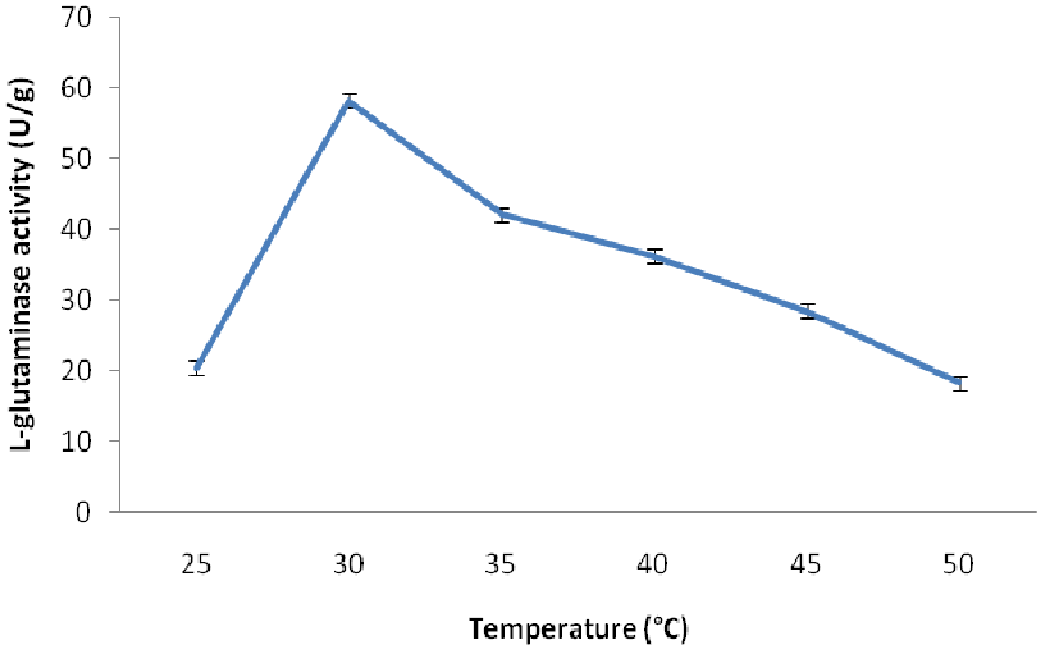

Figure 3. Effect of temperature on L-glutaminase production.

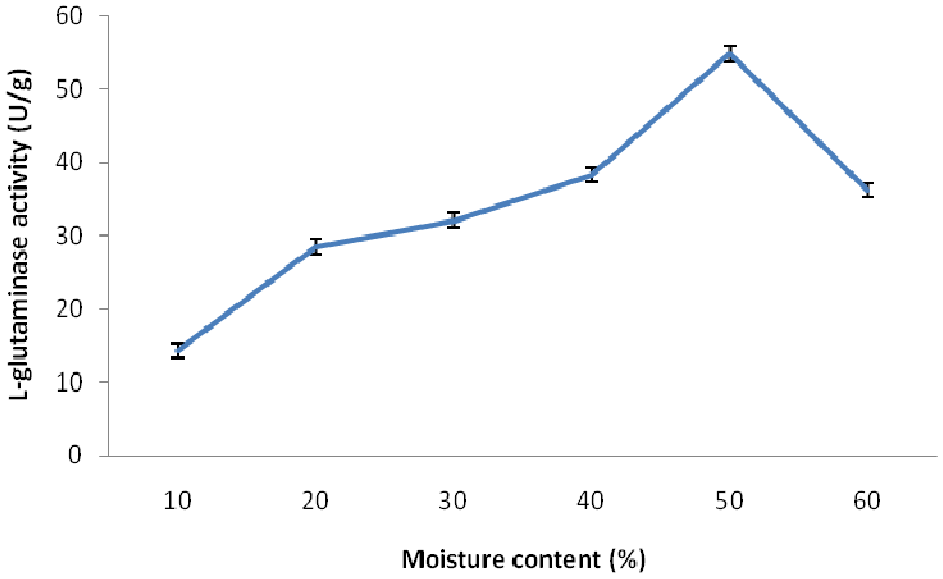

Figure 4. Effect of moisture content on L-glutaminase production.

suggesting that the end-point of fermentation should be carefully controlled because the synthesized enzyme could be degraded by non-specific proteases secreted by the fungus.

Effect of moisture content

Water added during inoculation was taken into account for the calculation of moisture content. In this study, six moisture levels ranging from 10 to 60% were established to study their effect on L-glutaminase production and the results obtained are shown in Figure 4. It could be seen that the maximum production of glutaminase (54.8 ± 0.24 U/g) occurred at 50%. The enzyme activities either decreased with further increase or were found to be low in moisture content. In SSF, microbial growth and product

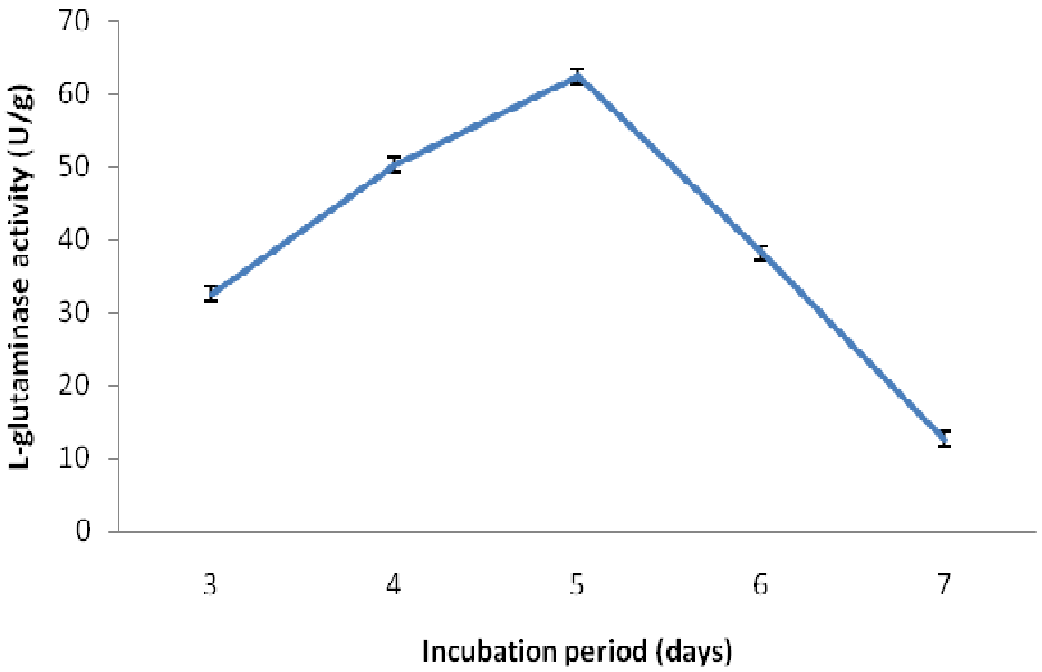

Figure 5. Effect of incubation period on L-glutaminase production.

formation occurs at or near the surface of the solid substrate particle having low moisture contents (Pandey et al., 1994). A reduction in the solubility of nutrients of the substrate and a low degree of swelling are the disadvantages of low moisture content.

Effect of incubation period

Incubation period was the most important physical variable in the SSF. To determine the optimum incubation period for L-glutaminase production, fermentation flasks were incubated for different time duration (3 to 7 days). Enzyme activity was analyzed at every 24 h time intervals (Figure 5). With regard to the different incubation periods, the maximum enzyme production was found to be 62.47 ± 0.32 U/g on the 5th day of incubation. Further incubation after this temperature did not show any increment in the enzyme production level. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature (Tunga et al., 1999).

Effect of carbon sources

Extracellular enzyme production depends greatly on the composition of the medium. The mineral salt solution for glutaminase had glucose as the sole carbon source.

When glucose was replaced by various sugars, dextrose was found to be an effective carbon source for glutaminase production (Figure 6). The enzyme activity was highest (60.42 ±0.56 U/g) when dextrose was supplied at a concentration of 1% w/v. Similar results were observed for the effect of glucose on L-glutaminase production by *Aspergillus oryzae*, *Vibrio costicola, Beauveria bassiana,* and *T. koningii* (Yano et al., 1988; Prabhu and Chandrasekaran, 1996; Sabu et al., 2000; El-Sayed, 2009).

Effect of nitrogen sources

Since any biotechnological process is likely to be based on crude enzymes, selection of best nitrogen sources is important to increase their activities in the culture supernatants. As far it is concerned, nitrogen can be an important limiting factor in the microbial production of enzymes. As shown in Figure 7, among the different organic sources tested, yeast extract (1% w/v) was the best source for maximal enzyme production (56.42 ± 0.57 U/g). These results correlate with the results of *T. koningii* for L-glutaminase production (El-Sayed, 2009).

Effect of metal ions

Incorporation of additional metal ions played a significant role in the enzyme production. However, the requirements of specific metal ions depend on the sources of enzymes. The maximum enzyme produced was

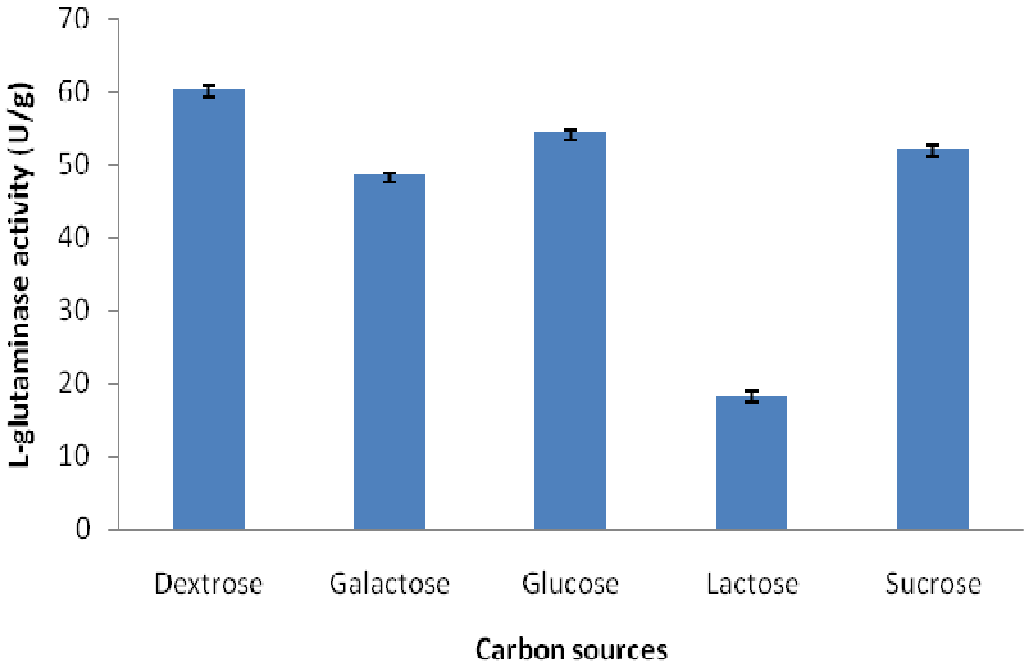

Figure 6. Effect of additional carbon sources on L-glutaminase production.

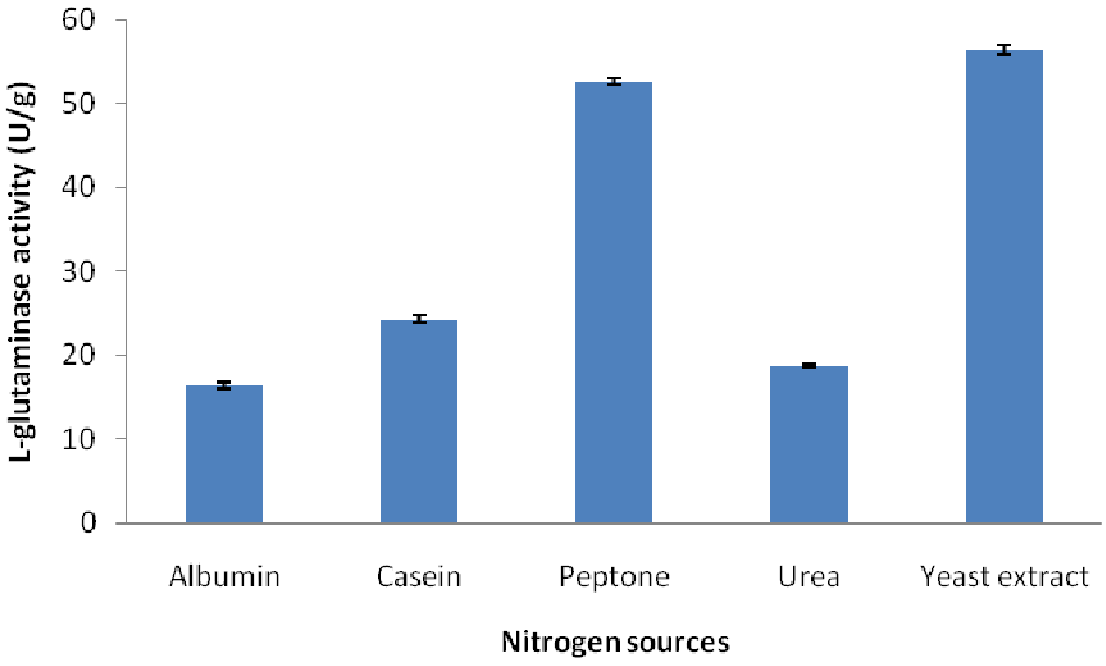

Figure 7. Effect of nitrogen sources on L-glutaminase production.

observed in flasks supplemented with $MgSO_4$ as nutritional factor (Figure 8). It showed a maximum enzyme productivity of 58.03 ± 0.35 U/g. This suggests that these metal ions are detrimental to the improvement of glutaminase production. Similar results were also reported for glutaminase production (Prabhu and Chandrasekaran, 1996). These results can be attributed to the fact that the metals might be acting as cofactors for

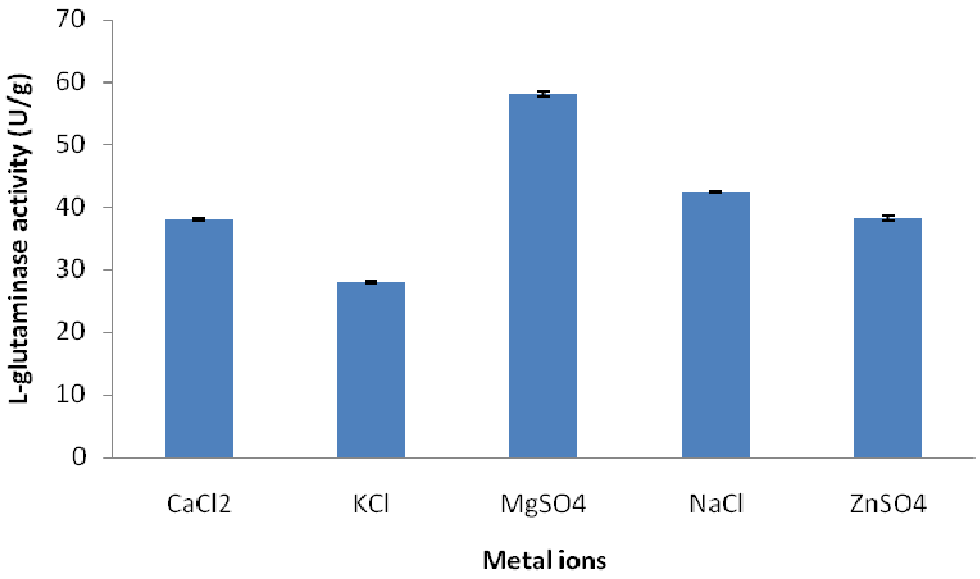

Figure 8. Effect of metal ions on L-glutaminase production.

many enzymes involved in intermediatory metabolism (Jellison et al., 1997).

Conclusion

This study has been taken up with a view of exploring the possibilities of using tea dust as a substrate and *A. flavus* as a microbial source for the production of L-glutaminase. The results obtained in this study revealed the potential of *A. flavus KUGF009* as a source of L-glutaminase which recently has gained significance in pharmaceutical and food industry. The use of inexpensive agro-industrial wastes will have important economic advantages over submerged fermentation. To the best of our knowledge the yield reported is the highest by this fungus using SSF.

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