Research Article

Influence of Fermentation Process Parameters on L-Glutaminase production by *Bacillus subtilis* RSP-GLU

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ABSTRACT
An L-glutaminase producing microorganism was isolated and identified as *Bacillus subtilis* RSP-GLU. The production pattern of the enzyme by isolated microorganism was studied in submerged fermentations (Smf). Initially various process parameters such as pH, incubation temperature, inoculum concentration and agitation speed were optimized based on the one-at-a-time method. It was noticed that at pH 7.0 is the optimum for L-glutaminase production. The maximum production of the enzyme was observed at temperature 37 ^{0}C, initial inoculum concentration 2% and agitation speed 100 rpm grown culture broth at 24 hours of incubation. At the optimized conditions0.682 g L^{-1} of biomass and 196 U m^{-1} of enzyme was obtained.

Keywords: L-Glutaminase, Submerged fermentation, Anti leukemia, Optimization, One at a time.

INTRODUCTION

L-glutaminase (EC 3.5.1.2) is an enzyme has substantial contributory role in cellular nitrogen metabolism in all living cells[1]. It catalyzes the conversion of L-glutamine to L-glutamic acid and ammonia. It is an important enzyme in both pharmaceutical and food industrial sectors. L-glutaminase is an effective agent in the treatment of acute lymphocytic leukemia and HIV. L-glutaminase mediated selective leukemia cell death mainly associated with non-availability of L-glutamine for protein synthesis as well as associated amino acids metabolism. Exogenous supply of L-glutaminase depletes the L-glutamine levels in the intracellular pool of blood. Since the cancerous cells unable to synthesis L-glutamine due to the lack of L-glutamine synthase unlike normal cells which causes L-glutamine mediated selective death of leukemic cells. It is also used as an analytical agent, as biosensing agent, as a flavour-enhancing agent and in the production of specialty chemicals like threonine by its gamma glutamyl transfer reactions[2]. Based on industrial and therapeutic use of L-glutaminase, the scientific community encouraged to explore the different resources for effective production of this enzyme.

The microbial production of metabolites and enzymes mainly depend on the genetic nature of the organism, fermentation medium components and their concentration, physiological growth conditions and interactive influence of all the above factors. Hence optimization of the above conditions is vital in order to get higher yields and to develop effective bioprocess system for industrial application. Many authors reported that the increased enzymes yield upon optimization of bioprocess conditions using different fermentation strategies[1-4].

Development of ideal medium requires pre-requisite information of key parameters and their optimum concentration level of different fermentation parameters related to nutritional, physiological and biological parameters[4-7]. The authors were isolated a high yielding L-glutaminase producing microorganism and identified as *Bacillus subtilis* based on ribotyping[2]. In the preliminary stage, hence investigation was performed to develop a suitable fermentation medium for isolated *Bacillus subtilis* RSP-GLU for L-glutaminase production by studying the effect of various physiological and culture conditions.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Isolated *Bacillus subtilis* RSP-GLU MTCC 9727 (Accession No. AM990996) was used in this study. The culture was maintained on L-glutamine medium (pH -7.0) slants consisting of (g L^{-1})glutamine -5, K_2HPO_4 -1, KH_2PO_4-0.1, $MgSO_4$ -1, NaCl -0.5, yeast extract -0.5 and agar -20. Inoculated slants were

incubated at 35 ^{o}C for 24 h for microbial growth and were stored at 4 ^{o}C in a refrigerator for further use.

Optimization of L-glutaminaseProduction in submerged fermentation

The enzyme production experiments were performed in 250 ml conical flasks containing 100 ml of medium containing (g L^{-1})glutamine -5, K_2HPO_4 -1, KH_2PO_4-0.1, $MgSO_4$ -1, NaCl -0.5, yeast extract -0.5. In order to optimize the various process parameters such as pH, temperature, inoculum size and agitation speed were varied. After fermentation the broth wascollected and centrifuged at 10000 rpm at 4 ^{o}C for 10 min and supernatant cell free broth was used as enzyme source. The production and biomass was monitored in whole experiments and a comparative study was performed. All experiments were performed in triplicate and the average values are reported in the present study.

Estimation of L-glutaminase activity

L-glutaminase activity was determined using L-glutamine as a substrate and the product, ammonia, released during the catalysis was measured by using Nesseler's reagent according to method of Imada*et al*[8]. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 Mol of ammonia under optimal assay conditions.

Estimation of Biomass

The fermentation medium was taken in a regular time intervals and the absorbance of medium was measured at 600nm. From the absorbance the dry weight was calculated by using the standard curve of absorbance vs. dry weight which was constructed earlier.

RESULTS AND DISCUSSION

The role of medium pH on L-glutaminase production

pH of the growth medium is reported to influence the growth of any microbial strain and subsequent metabolic product formation in addition to its secretion in case of extracellular products[1-2]. In general, L-glutaminase production by most of the microbial organisms under submerged fermentation conditions is extracellular in nature and observed to be produced optimum in the pH range 6.0 to 8.0[1-2]. Therefore, the imperative role of the pH of the medium on L-glutaminase production by this isolated *Bacillus subtilis* RSP-GLU was characterized by studying the enzyme production pattern in different pH conditions ranging from 4.0 to 9.0. It was noticed that pH at 7.0 is the optimum (160 U ml^{-1}) for the production of the L-glutaminase and any further alteration either increase or decrease of the medium pH negatively influenced the enzyme production (Fig. 1). These results are in consistent with literature reportson the enzyme production where effective enzyme yields noticed in the pH range of 6.0 to 8.0. However,theL-glutaminase production has also been noticed in the wide range of pH conditions, from pH 5.0 to 9.0 depending on the nature of organism [9-11].

Critical analysis of theL-glutaminaseproductivity values do suggest that alkaline conditions are more supportive compared to the acidic environment during fermentation by this isolate. This could be confirmed on the basis of observation that a variation of one unit of pH towards alkaline side i.e. from pH 7.0 to 8.0, the enzyme yield is reduce only by 39 % while the same shift towards acidic side (pH 7.0 to 6.0) resulted in 47 % reduction of enzyme production, to that observed at pH 7.0 (160 U ml^{-1}) (Fig 1) indicating a variation of enzyme production.

Most of the metabolism linked enzyme production by any microbial strain is always growth associated. To evaluate the same in case of L-glutaminase production by this *Bacillus subtilis* RSP-GLU the biomass and L-glutaminase yields with the time line of fermentation was investigated in all selected pH conditions. Analysis of the data denoted that biomass production was directly proportional to L-glutaminase suggesting the enzyme production in this microbial strain is growth associated and both L-glutaminase and biomass production is regulated by the medium pH. Both biomass and enzyme production maxima was noticed at pH 7.0. At this pH value, the biomass production was observed to 0.58 g L^{-1} (Fig.1). Either increase or decrease the pH the biomass production was decreased. The biomass at pH 6.0 was observed to be (0.31 g L^{-1}) which is 48.0 % less than the biomass obtained at neutral pH whereas increasing the pH from 7.0 to 8.0 resulted 40.0 % decreased biomass was noticed. The observed data trend is in accordance with literature reports, where it was noticed that the pH optimum is organism specific. This could be evidenced from that observation that *Beauveria* sp. BTMF S10 and *Streptomyces rimosus*showed growth and enzyme production at the optimum pH of 9.0[10], whereas*Stenotrophomonasmaltophilia* NYW-81 produce the maximum L-glutaminase at acidic conditions (pH 6.0)[11]. The observed difference of biomass production with the change of fermentation medium pH change

along with L-glutaminase enzyme production variations further confirm that this enzyme production is growth associated in this bacterial strain.

Effect of incubation temperature on L-glutaminase production

Incubation temperature dependent variation in L-glutaminase production was reported in several microbial species[10,11]. Keeping this in view, experiments were performed to understand the effect of fermentation incubation temperature the onL-glutaminase production by the isolated bacterial strain by incubating the fermentation medium at different temperatures ranging from 33 to 41^{o}C. Figure 2 revealed that the incubation temperature regulated L-glutaminase production by *Bacillus subtilis* RSP-GLU. Parabolic nature of enzyme production curve was noticed with increase in incubation temperature. Maximum L-glutaminase production of 167 U ml^{-1} was noticed at a temperature of 37 ^{o}C. Variation of the temperature in either side of this resulted in decrease of L-glutaminase production in cell free broth. The loss of activity is more at the higher temperature when compared to the lower temperatures. Increase of 2 ^{o}C temperature from 37 to 39 ^{o}C caused 56.0 % reduction in enzyme production. Whereas 30.0 % of enzyme production decreased was noticed due to change of the temperature from 37 to 35 ^{o}C.

Analysis of the biomass data revealed that the highest biomass production (0.612 g L^{-1}) was observed at 37 ^{o}C. The biomass production profiles also trail the similar pattern of the L-glutaminase production. Based on the literature, the optimum temperature for L-glutaminase production is varied with micro-organism used. It was observed that 27 ^{o}C is optimum for the enzyme production by *Beauveria* sp. BTMF S10 and *Streptomyces rimosus*[9,10]. However, the high amount of L-glutaminaseby*S. maltophilia* NYW-81 was reported at 30 ^{o}C[11].

Influence of initial inoculum level on L-glutaminase production

Quantum of initial biomass controls the kinetics of growth and several biological metabolic functions leading to the overall biomass and extracellular product production[12]. To study the same, experiments were planned with increasing inoculum concentration from 1.0 to 3.0 % and the L-glutaminase activity was monitored during growth phase of isolated *Bacillus subtilis* RSP-GLU. The results indicated that, L-glutaminase production kinetics varied with variation in initial inoculum level (Fig. 3). The maximum enzyme production (176 U ml^{-1}) was observed in 2.0% of initial inoculum supplemented conditions. Reduction of inoculum concentrationcaused lower enzyme production but this reduced enzyme production was minimal at 1.5 % inoculum concentration. At this concentration the L-glutaminase production was observed to be 142 U ml^{-1} which is 19.0 % lesser than the optimum enzyme production (176 U ml^{-1}). At 1.0 % inoculum concentration the L-glutaminase production was found to be 85 U ml^{-1} which is 51.0 % lesser than the enzyme produced at optimum inoculum concentration 2.0%. However, supplementation of higher concentration of inoculum, over the optimum (2.0 ml) also resulted in major reduction of the enzyme and biomass production. This could be evidenced from the observed data at 2.5 ml of inoculum supplementation, only 100 U ml^{-1} of enzyme production was observed which is 43.0% lesser than the L-glutaminase produced with 2.0 ml of inoculum (Fig. 3). Such a high inoculum dependent enzyme repression was also observed with other L-glutaminase producing microbial species in solid-state fermentation[13,14]. The production of biomass also follows the similar pattern of enzyme production. From the Figure 3 the highest biomass production 0.634 g L^{-1} was observed with 2.0 ml of inoculum concentration. Either side of these concentration decreased biomass production was observed.

Role of agitation on L-glutaminase production

Understanding of mass transfer of substrates, products, nutrients and gases among the system components is one of the important parameters to be considered for optimal biotechnological production of any metabolite or product and exploitation of microbial capability under fermentation process[15-16]. This is basically achieved in various laboratory scale experiments by agitating the culture components in controlled environmental set up. To evaluate the impact of agitation on L-glutaminase production by this *Bacillus subtilis* RSP-GLU, experiments were carried out in different agitation conditions ranging from 80 to 130 rpm with 10 units variation and the L-glutaminase production kinetics were followed. The data indicated that the production of L-glutaminase was influenced by agitation levels of fermentation broth (Fig. 4). Maximum enzyme activity (196 U m^{-1}) and biomass production (0.682 1 g L^{-1}) were observed at

100 rpm grown culture broth at 24 hours of incubation.

Analysis of the relation between productivities and aeration levels as rpm depicted that change in agitation speed in either side of 100 rpm resulted in drop of enzyme activity as well as biomass production. The losses of the biomass and enzyme activity were high when the rpm was decreased. It was observed that at 90 rpm the biomass (0.449 g L^{-1}) and L-glutminase production (150 U m^{-1}) which were 34.0 % and 23.0 % less thanobserved optimum at 100 rpm, respectively. From the Figure 4 it was inferred that at higher rpm the reduction of biomass and L-glutaminase production was less when compared at lower rpm. A 69.0 % and 57.0 % reduction of the biomass and enzyme production wereobserved at 80 rpmrepectively. Whereas at 120 rpm 44 % and 33 % reduction in biomass and L-glutaminase yield was noticed when compared with the biomass and enzyme production at 100 rpm. These results suggested that for effective production of L-glutaminase by isolated *Bacillus subtilis* RSP-GLU requires a proper aeration and agitation.

Overall, L-glutaminase production with this isolate, *Bacillus subtilis* RSP-GLU is regulated by several bioprocess parameters and the enzyme is growth associated. A maximum of 196 U ml-1 enzyme production yields were achieved with the optimization of medium pH, incubation temperature, inoculum concentration and aeration level as rpm. Both biomass and enzyme production parameters were influenced by above parameters and any variation in optimized value caused reduction in L-glutaminase as well as *Bacillus subtilis* RSP-GLU biomass production.

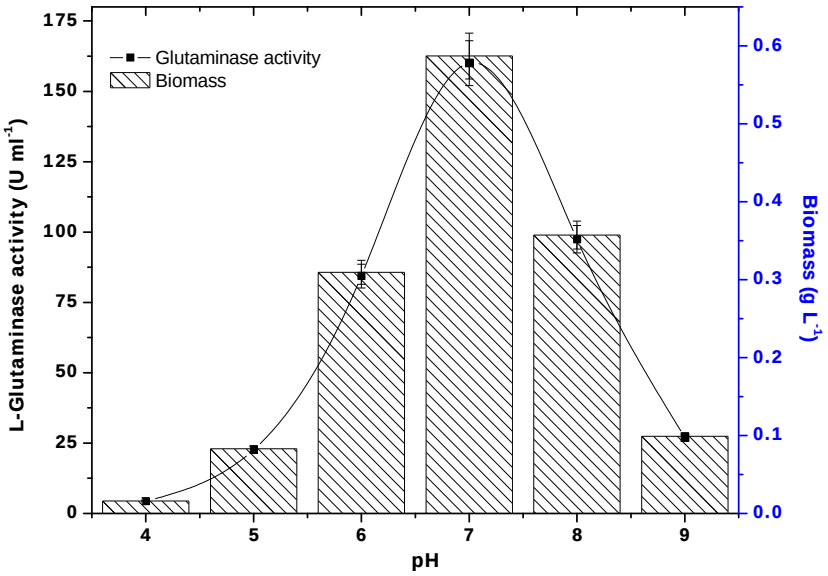

Fig. 1: Effect of pH on L-glutaminase production

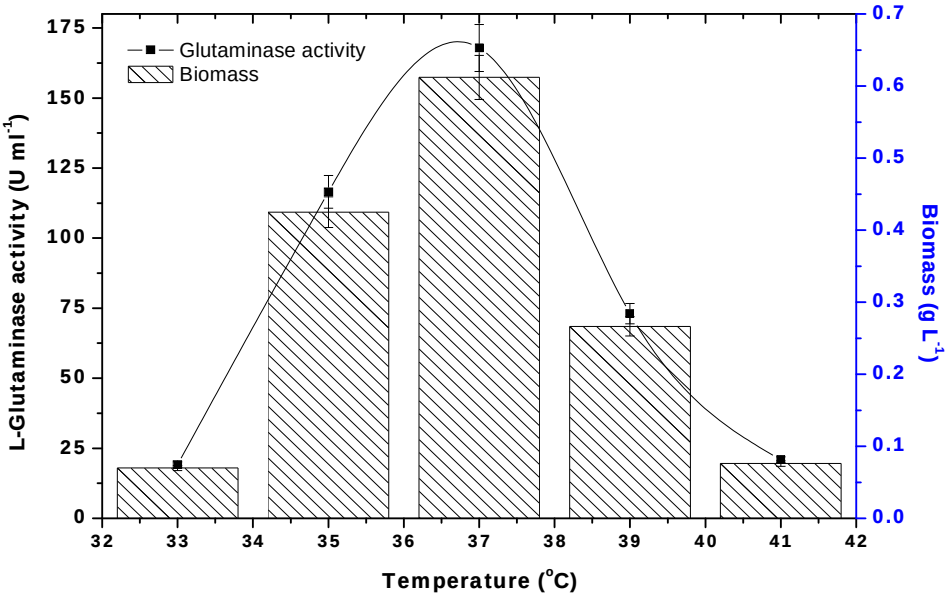

Fig. 2: Effect of temperature on L-glutaminase production

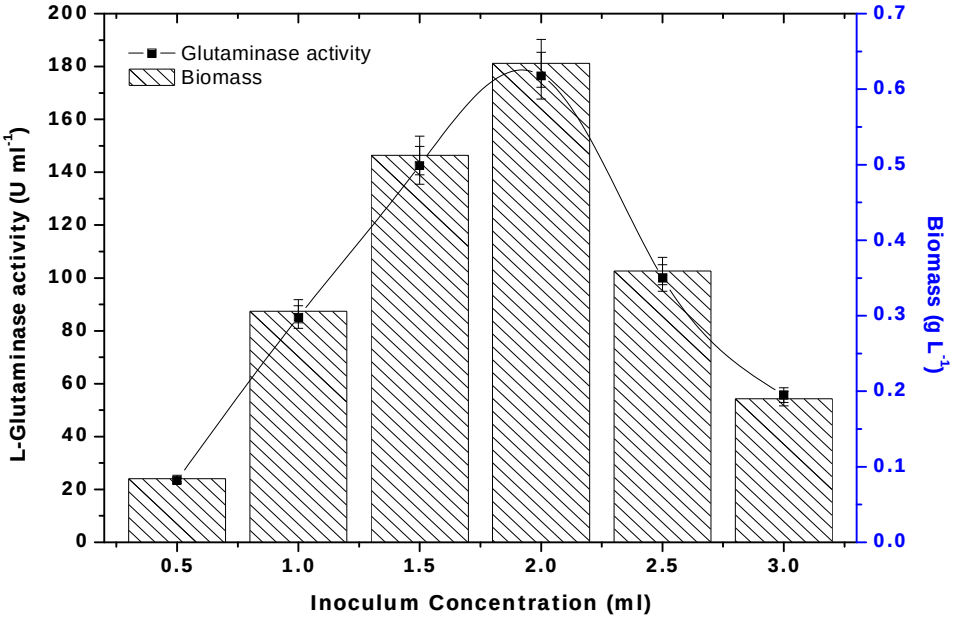

Fig. 3: Effect of initial inoculum concentration on L-glutaminase production

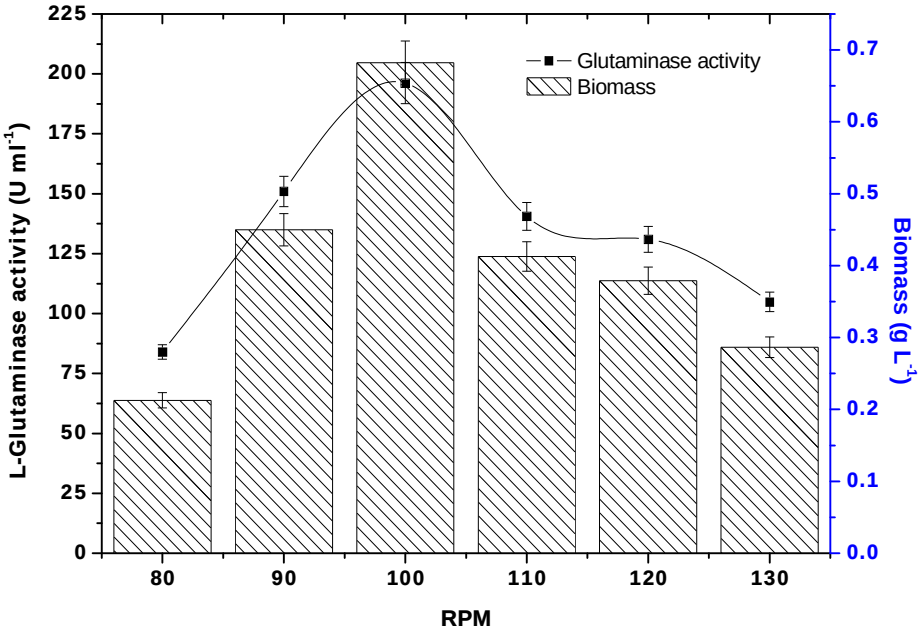

Fig. 4: Effect of RPM on L-glutaminase production

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