INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
SHORT ABSTRACT OF THESIS

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Thesis Title: *Bacillus subtilis* as an expression host for the production of glutaminase free recombinant L-asparaginase II

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SHORT ABSTRACT

The bacterial L-asparaginase has been widely used as a therapeutic agent in the treatment of ALL (acute lymphoblastic leukemia). Moreover, it is used in food industry for the production of acrylamide free starchy/baked foods, L-asparagine biosensor for diagnosis of leukemia and as a model enzyme for the development of new drug delivery systems. The various side effects of L-asparaginases are mainly due to the presence of partial glutaminase activity. Hence, we made an attempt to produce glutaminase-free L-asparaginase II, which is highly desirable for its successful application.

The gene encoding glutaminase-free L-asparaginase II (*ans B*2) from *Pectobacterium carotovorum* MTCC 1428 was cloned into pHT43 vector and transformed in *Bacillus subtilis* WB800N. It was further optimized to maximize the expression levels of recombinant enzyme (rL-asp II). A three-fold higher enzyme production was observed with an efficient transformant as compared to native strain. Enzyme localization studies revealed that > 90 % of recombinant enzyme is secreted extracellularly. The expression of recombinant L-asparaginase II was confirmed by SDS-PAGE, IMAC (Immobilized metal ion affinity chromatography) purification followed by Western blotting. Process parameter optimization with OFAT (one factor at a time) revealed that an agitation (120 rpm), temperature (37 ºC), Isopropyl b-D-1-thiogalactopyranoside (IPTG) concentration (1 mM) and time of induction (at 0.8 OD600nm) plays a vital role in achieving a maximum of 55 IU/ml. Furthermore, consecutive induction by IPTG improved the enzyme production up to 105 IU/ml of protein.

The crucial components, which elevate the expression of rL-asp II from *Bacillus subtilis* WB800N were analysed. The Plackett-Burman tool identified sucrose, NH_4Cl, NaH_2PO_4 and $MgSO_4$ as the significant influencing factors ($p<0.05$). Further investigations showed that artificial neural network genetic algorithm (ANN-GA) was more effective than central composite design (CCD) in optimizing the influencing factors. The maximum rL-asp II expression was found to be 389.56 IU/ml and 525.98 IU/ml using CCD (R^2=90.4%) and ANN-GA (R^2=96.2%), respectively. The validation experiments were carried out in a 3 L batch bioreactor where kinetic modelling of the obtained data was done.

The possible effect of growth inhibition caused due to initial sucrose concentration on *Bacillus subtilis* WB800N for the production of rL-asp II was evaluated. The experiments were carried out at different initial substrate concentrations of sucrose (2.5 - 180 g L^{-1}). The results obtained were used to develop and analyze the biokinetic growth models. Among different concentrations, 40 g L^{-1} of sucrose showed highest specific growth rate of 0.125 h^{-1}. Employing various 3 and 4 parametric Monod-variant models estimated that the 4- parameter growth model proposed by Luong was found to be best fit, to describe the dynamics for sucrose consumption and growth kinetics of *Bacillus subtilis* WB800N (R^2=0.979). The non-linear regression analysis was applied to estimate the model parameters as well as goodness of fit. The optimization of model parameters using parameter sensitivity analysis revealed that μ_{max}, K_I, and γ_S^* are the most sensitive parameters in modeling of growth inhibition kinetics of recombinant *Bacillus subtilis* WB800N.

The purified rL-asp II showed no glutaminase activity, which may reduce the possibility of side effects of the enzyme during the course of anti-cancer therapy. The rL-asp II from *B. subtilis* WB800N was purified using His-tag column. The yield of purified enzyme was found to be 42.91 IU indicating 86.9 % recovery with purification fold of 3.79. The molecular modelling of the L-asparaginase II was performed to analyze the residues involved in active site formation. The L-asparaginase II sequence was modelled using the modeler 9.13 software. The structure-based sequence alignment of 1HG1.A, 2JK0.A pdb structures with modelled protein showed sequence similarity of 67.5 % and 67.3 % while the percentage of secondary structure similarity was observed to be 88 % and 87 %, respectively. The absence of GLU63 in rL-asp II was found to be the main reason behind the no glutaminase activity. It predicted that amino acids, GLY60, GLY119 and ALA252 in the active site are responsible for the glutaminase free L-asparaginase II activity.

The rL-asp II enzyme is very specific towards its natural substrate, L-asparagine. The activity of rL-asp II was activated by mono cations and various effectors including Na^+, K^+, L-histidine, L-cystine, glutathione and 2-marceptoethanol whereas it is moderately inhibited by various divalent cations and thiol group blocking reagents. Kinetic parameters, K_m, V_{max} and k_{cat} of the rL-asp II were found to be 0.65 mM, 4.018 IU μg^{-1} and $2.496 \times 10^3\ s^{-1}$, respectively. Optimum pH of the purified enzyme for the hydrolysis of

L-asparagine was in the range of 8.0 – 9.0, and its optimum temperature was found to be 40°C. The minimum deactivation rate constant (k_d) and maximum half life ($t_{1/2}$) were found to be 0.043 min^{-1} and 16.1 h, respectively at pH of 8.6 and 40ºC. Thermodynamic parameters (ΔG^*, ΔH^*, ΔS^*, and activation energy) were also evaluated for purified rL-asp II. The probable deactivation mechanism of rL-asp II was described in this study. The fluorescence spectral studies reveal that the enzyme retains its secondary structure at 40ºC. In addition, the effect of rL-asp II on acrylamide reduction was evaluated. It was observed that the acrylamide content of potato chips using mustard oil reduced up to 95 % by using 10 IU/ml of rL-asp II per gm of potato chips. *In vitro* anti-cancer activity assays using three different human cancer cell lines (HeLa, SAS and MCF 7) were done to evaluate the therapeutic potential of rL-asp II.