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# Enhanced production of glutaminase free L-asparaginase II by *Bacillus subtilis* WB800N through media optimization

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**Abstract**−We studied the crucial components which elevate the expression of recombinant novel glutaminase free L-asparaginase II (rL-asp II) from *Bacillus subtilis* WB800N. 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 rL-asp II expressed effectively inhibiting the polyacrylamide formation *in vitro* where no solidification was observed, when 2 ml of purified rL-asp II used even after 60 min of incubation. This is the first study to report highest production of rL-asp II in *B. subtilis* WB800N (525.98 IU/ml) till date by combining statistical designs with consecutive intermittent addition of IPTG in batch reactor.

Keywords: *Bacillus subtilis* WB800N, L-asparaginase II, Artificial Neural Network-genetic Algorithm, Batch Reactor, Unstructured Bio-kinetic Models

## INTRODUCTION

L-asparaginase is an amidohydrolase (E.C. 3.5.1.1) which cleaves the amino acids to its respective acid and amino group. It is produced/found in a wide range of organisms, such as bacteria, fungi, serum of guinea pigs [1-4] and rodents, but is absent in *Homo sapiens* [5]. L-asparaginase has been isolated from various sources [6-8], but only asparaginase produced by *E. coli* and *Erwinia chrysanthemi* were used in clinical use [9,10]. Microbially produced L-asparaginases were preferred over animal or plant sources due to their ease of process operation/modification, optimization, economic consistency and purification [11-13]. Microbial L-asparaginases are more stable than L-asparaginases from other sources. Hence, microbial sources are best for the bulk production of enzymes especially for clinical use [14,15]. The main drawback of the L-asparaginase enzyme in clinical use is its ability to cause side effects such as allergies, neurotoxicity, hepatotoxicity, impairments in blood coagulation and anaphylactic shocks [16,17]. The partial glutaminase activity of L-asparaginase enzyme is a reason for its side effects. Therefore, the need of the moment is to find a novel enzyme with low/or no glutaminase activity, higher therapeutic activity and different serological properties. In our previous study, we cloned and expressed glutaminase free L-asparaginase II enzyme from *Pectobacterium carotovorum* MTCC 1428 in *Bacillus subtilis* WB800N [18]. Apart from clinical use, L-asparaginase is used in food based industries [19], where it aids in reduction of acrylamide in starch foods. It is also widely used as a potent therapeutic agent in the treatment of acute lymphoblastic leukaemia and non-Hodgkin lymphoma [20]. L-asparaginase is also used as biosensor in detecting the levels of L-asparagine in biological samples [21]. Some researchers found that by treating potato chips, French fries and dough biscuits with L-asparaginase II enzyme prior to frying/baking at high temperatures has reduced the acrylamide formation significantly, which is a cancer causing agent in humans [19,22-25].

Over the last few decades, good progress has been witnessed in the field of genetic engineering and cell engineering, where various genes have been cloned and expressed in eukaryotic and prokaryotic cells to enhance heterologous proteins production [26-28]. Recently *Bacillus subtilis* expression system has been much focused as an attractive cell factory for the heterologous expression of proteins, vitamins and antibiotics [29]. *Bacillus subtilis* is a GRAS (Generally regarded as safe) approved by FDA [30], endowed with unique features such as no codon bias and also has the potential of extracellular secretion of recombinant enzymes [31,32]. Recently, a study reported that *Bacillus subtilis* is used in the fermentation of soybeans (Japanese traditional food Natto) [33], which shows that this strain is more eco-friendly and can be used for the production of various heterologous proteins.

Till date, more than 60% of commercial proteins are being reported to produce by *Bacillus* strains [34-37]. It can grow on a wide variety of carbon, nitrogen sources and has the ability to reach high cell densities of 100 g dcw $l^{-1}$, 184 g dry cell weight $l^{-1}$ and 56 g dcw $l^{-1}$, respectively [38-40]. Hence, it is considered as the best strain for the production of glutaminase free L-asparaginase II. So optimization of media components for the enhanced production of proteins is a valuable addition to the current research of heterologous

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protein production [41-44]. Hence, a medium that supplies defined quantity of nutrients to the developed strain (*Bacillus subtilis* WB800N pHT43-*ans* B2) for the production of recombinant novel glutaminase L-asparaginase II is very crucial. To improve the supply of nutrients, the media components were modified from Wenzel et al. [45], through various statistical approaches [46].

Optimization studies involving a one factor at a time approach are not only tedious but time consuming, and hence it may overlook the interaction effect between the variables. In contrast, statistical methodologies are preferred due to their advantages such as reducing the number of experiments and interpretation of possible interactions among the factors and thus reducing error in an economical manner [46,47]. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes [48]. The most commonly used design in RSM is central composite design (CCD).

Apart from statistical approaches such as RSM, other mathematical approaches like artificial neural network (ANN) coupled with genetic algorithm (GA) have also gained much of the research interest, because of their ability to optimize nonlinear modelling problems with much more efficiency than previous methods [49-51]. The major advantage of ANN over the classical mathematical models is that they allow the simultaneous identification of structure parameters, while they possess the ability to adapt by examples [52]. The concept of genetic algorithms (GAs) is based on the evolutionary natural selection processes (Darwinism and Mendelism), where selection results, in species that fit the best among the population [51].

In the current study, we achieved high level of rL-asp II expression by optimizing the media components affecting the production with respect to our previous report, where we cloned the L-asparaginase II gene in *B. subtilis* WB800N [18]. To further improve the rL-asp II expression, we applied consecutive induction strategy at the batch reactor level, which showed significant improvement. However, this strategy also minimized the production of overflow metabolites such as acetic acid and acetoin, which are the major bottlenecks in the production of recombinant proteins [39,53,54]. The efficacy of the rL-asp II produced was checked through *in vitro* inhibition of polyacrylamide formation.

## MATERIALS AND METHODS

### 1. Chemicals and Reagents

All the chemicals purchased were of high-grade quality from Himedia. The antibiotics used for the culturing of strain include chloramphenicol and neomycin (Sigma-Aldrich).

### 2. Media Development for the Optimized Expression of rL-asp II

2-1. Strain and Media Formulation

*Bacillus subtilis* WB800N harboring *ans B2* in pHT43 (pHT43-*ans B2)* vector was used for the optimization of recombinant L-asparaginase II (rL-asp II) expression [18]. The maintenance and sub-culturing of recombinant strain was performed on LB media (yeast extract 5 g/L, tryptone 10 g/L and NaCl 5 g/L) with 100 μg/ml chloramphenicol for plasmid selection (pHT43-*ans B2*) and 100 μg/ml neomycin for strain selection (WB800N). The production of rL-asp II has been studied in mineral salt media modified from that of Wenzel et al. [45], which consists of $Na_2SO_4$ (2.0 g $L^{-1}$), $(NH_4)_2SO_4$ (2.68 g $L^{-1}$), $NH_4Cl$ (0.5 g $L^{-1}$), $K_2HPO_4$ (14.6 g $L^{-1}$), $NaH_2PO_4{\cdot}H_2O$ (4.0 g $L^{-1}$), $MgSO_4{\cdot}7H_2O$ (1.0 g $L^{-1}$), tryptophan (1 g $L^{-1}$) (as the strain is auxotroph for tryptophan), trace element solution (TES) 3 ml $L^{-1}$ with carbon source 5 g $L^{-1}$ for pre-culture and 25 g $L^{-1}$ carbon source for the fermentation. TES contains $CaCl_2$ (0.5 g $L^{-1}$), $ZnSO_4{\cdot}7H_2O$ (0.18 g $L^{-1}$), $MnSO_4{\cdot}H_2O$ (0.1 g $L^{-1}$), $Na_2$-EDTA (10.05 g $L^{-1}$), $FeCl_3$ (8.35 g $L^{-1}$), $CuSO_4{\cdot}5H_2O$ (0.16 g $L^{-1}$), and $CoCl_2{\cdot}6H_2O$ (0.18 g $L^{-1}$). All the media components were autoclaved separately and reconstituted aseptically prior to inoculation to avoid precipitation of media components.

2-2. Fermentation Conditions

The inoculum was prepared by inoculating a single colony of WB800N/pHT43-*ans B2* into 10 ml of pre-culture (aforementioned media composition) followed by incubation at 37 °C, 120 rpm for about 12 h. The overnight pre-culture was inoculated aseptically into 150 ml shake flasks with a production medium of 25 ml. The culture sample was induced by adding the IPTG consecutively and intermittently at different growth periods [18]. The samples were then collected at regular intervals of 12 h and measured for rL-asp II expression and dry cell weight (DCW). All experiments were performed in triplicate.

2-3. Initial Screening of Carbon, Nitrogen Sources and Their Effect on rL-asp II Expression

Eleven carbon sources (glucose, glycerol, sucrose, sorbitol, mannitol, galactose, maltose, lactose, gluconate, pyruvate, ethanol) and eight nitrogen sources ($NH_4SO_4$+$NH_4Cl$, $NH_4SO_4$, $NH_4Cl$, glycine, L-asparagine, peptone, casaminoacids, yeast extract) were screened based on the expression of rL-asp II in the above-mentioned media.

2-4. Screening of Crucial Medium Components by Plackett-Burman Design

Plackett-Burman design was employed to screen the significant media components affecting the rL-asp II expression [55]. A total of eight parameters, sucrose, $NH_4Cl$, $Na_2SO_4$, $K_2HPO_4$, $NaH_2PO_4{\cdot}H_2O$, $MgSO_4{\cdot}7H_2O$, tryptophan and TES (trace element solution), were considered for the screening variables based on the previous reports [56]. The data range was varied based on the original medium components and represented at two levels, i.e., high and low which are denoted by (+1) and (−1), respectively, as shown in Table 1. Twelve experiments were performed in triplicate and the

**Table 1. Experimental variables at different levels used for the expression of rL-aspII by *Bacillus subtilis* WB800N/pHT43-*ans* B2 using Plackett-Burman design**

| Variables | Symbol code | Experimental values (g/L) | |
|---|---|---|---|
| | | Lower (−1) | Higher (+1) |
| Sucrose | X1 | 5 | 50 |
| $NH_4Cl$ | X2 | 1 | 6 |
| $Na_2SO_4$ | X3 | 1 | 5 |
| $K_2HPO_4$ | X4 | 5 | 25 |
| $NaH_2PO_4$ | X5 | 1 | 8 |
| $MgSO_4$ | X6 | 0.5 | 2.5 |
| Tryptophan | X7 | 0.5 | 2.5 |
| TES | X8 | 1 | 5 |

**Table 2. Experimental codes, ranges and levels of the independent variables for RSM experiment**

| Variables | Symbol coded | Range and levels | | | | |
|---|---|---|---|---|---|---|
| | | $-2(-\alpha)$ | −1 | 0 | +1 | $+2(+\alpha)$ |
| Sucrose (g/L) | X1 | 17.5 | 5 | 27.5 | 50 | 72.5 |
| $NH_4Cl$ (g/L) | X2 | 1.5 | 1 | 3.5 | 6 | 8.5 |
| $NaH_2PO_4$ (g/L) | X5 | 2.5 | 1 | 4.5 | 8 | 11.5 |
| $MgSO_4$ (g/L) | X6 | 0.5 | 0.5 | 1.5 | 2.5 | 3.5 |

average of results was taken as the response. The observed and predicted values are given in Table 3. The significance of each variable was determined by student's t-test. Plackett-Burman experimental design is based on the first order polynomial model, which is given as:

$$Y_{rL\text{-}asp\ II}=\beta_0+\Sigma\beta_i X_i \quad (1)$$

where $Y_{rL\text{-}asp\ II}$ is the response (rL-asp II activity), $\beta_o$ is the model intercept, $\beta_i$ is the linear coefficient and $X_i$ is the level of the independent variable. The significance of each variable was determined by student's t-test.

**3. Optimization of Crucial Medium Components Using Different Statistical Designs**

3-1. Response Surface Methodology (RSM)

Central composite design (CCD) was adapted with the view of further optimizing the screened variables and to explain the combined effect of the variables *viz*., sucrose, $NH_4Cl$, $NaH_2PO_4$ and $MgSO_4$ on rL-asp II production [57]. The screened significant variables and their ranges are given in the Table 2, where each of the variables was assessed with five coded levels (−2, −1, 0, +1, +2). According to CCD, 30 ($=2^k+2k+6$) experimental combinations were performed, where $k$ is the number of independent variables [58,59]. Twenty-four experiments with six replications at the center points were augmented to evaluate the pure error. All experiments were performed in triplicate and the average values with standard errors are reported in Table 5.

The data obtained from CCD on rL-asp II expression was subjected to analysis of variance (ANOVA). The second-order polynomial for predicting the optimal levels was expressed according to Eq. (2).

$$Y_{rL\text{-}asp\ II}=\beta_0+\Sigma_{i=1}^{k}\beta_i X_i+\Sigma_{i=1}^{k}\beta_{ii}X_i^2+\Sigma_i\Sigma_j\beta_{ij}X_iX_j \quad (2)$$

where $Y_{rL\text{-}asp\ II}$ is the predicted rL-asp II expression, k is the number of factor variables. $X_i$ and $X_j$ are independent variables, $\beta_0$ is the offset term, $\beta_i$ is the $i^{th}$ linear coefficient, $\beta_{ii}$ is the $i^{th}$ quadratic coefficient, and $\beta_{ij}$ is the $ij^{th}$ interaction coefficient. The statistical significance of the model equation and the model terms was evaluated by Fisher's test. The model efficiency was expressed by the coefficient of determination ($R^2$). Regression analysis and response surface graphs was plotted with the aid of statistical software package MINITAB® Release 16.1.1, PA, USA.

3-2. Artificial Neural Network Linked Genetic Algorithm (ANN-GA)

In this protocol, we employed feed forward back propagation method to train the network. The input and output of CCD was used as input and output neurons. The network architecture consists of four input layers: sucrose, $NH_4Cl$, $NaH_2PO_4$ and $MgSO_4$, three hidden layers and one output layer representing rL-asp II expression (Fig. 4(a)). In feed forward system, the data flows from input layer to output layer by the hidden layer. The input, hidden and output layers are connected with weights (w) (real number quantity associated with the connection between two neurons) and biases (b) that are considered parameters of the neural network (NN) [60,61]. The system performs its work by summing up all weighted inputs including biases and transfers it to the first nonlinear transfer function (tansig), which is situated between the input and hidden layer. The output produced by the hidden layer will then be transferred to the linear transfer function, namely purelin, from where the output will be transferred to the output layer.

$$\text{Purelin}=\text{sum} \quad (3)$$

$$\text{tansig}=\frac{1+\exp(-\text{sum})}{1-\exp(-\text{sum})} \quad (4)$$

The total experimental data was divided into three different sets: 20, 5 and 5 of data sets were used as training, validation and testing, respectively. The error function is calculated based on the difference between actual output and predicted output. ANN is an iterative method, which is pre-specified to minimize error function and adjust weight appropriately. The commonly used error functions, i.e., mean squared error (MSE) was used in the present study and is given by Eq. (5):

$$\text{MSE}=\frac{1}{N}\sum_{i=1}^{N}(Y_a-Y_p)^2 \quad (5)$$

where $Y_a$ is the actual output, $Y_p$ is the predicted output and N is the number of data points. The MSE is minimized by adjusting the weights and biases appropriately. During the training step, the weight and biases are iterated by Levenberg-Marquardt algorithm, until the convergence to the certain value is achieved. In this work, a Neural Network Toolbox of MATLAB (2010a) mathematical software was used to predict the rL-asp II expression [62].

3-3. Genetic Algorithm (GA)

The genetic algorithm (GA) is a global optimization procedure, where the convergence is independent of the initial value. Once the ANN is developed, the input space is further optimized using GA. GA follows four steps to find global solution. In the first step, initialization of the solution for the population will take place followed by fitness computation, which in turn is dependent on an objective function, and in the next step the better individual will be selected. The selected individual will then undergo crossing over and mutation, which leads to the creation of new sets of individuals with better performance. This process will be repeated until a maximum output result was achieved [60].

3-4. Unstructured Bio-kinetic Modelling for Batch Fermentation Studies

Inoculum for the bioreactor was prepared using the previously mentioned modified mineral salt medium. The preculture was prepared by inoculating sterile medium (100 mL) with loop of recombinant *Bacillus subtilis* WB800N/pHT43-*ans B2* in a 250-mL Erlenmeyer flask as explained above. Later the flask was incubated at

37 °C, 120 rpm for 16 hrs. The entire content of the flask with an average $O.D_{600\,nm}$ (optical density) of 5-6 was used for inoculating the bioreactor. Batch fermentations were performed using a 3 L ez-control (Applikon, Netherlands) stirred tank bioreactor. Initially, the bioreactor contained 1 L of the liquid medium. The incubation temperature and the agitation speed were maintained at 37 °C and 300 rpm, respectively. The aeration rate was 1.5 L $min^{-1}$. The dissolved oxygen level was maintained at 30% of the air saturation value.

Kinetic modelling is regarded as an indispensable step while developing an industrial fermentation process, since it helps to determine the optimal operation conditions for the production of the target metabolite. The growth analysis was done using a logistic equation, whereas, the Luedeking-Piret equation and the Modified Luedeking-Piret equation were used for analysis of product formation and substrate utilization respectively [63].

3-4-1. Logistic Equation

The exponential growth phase can be characterized by the following first-order equation which states that the rate of the increase of cell biomass is proportional to the quantity of viable cell biomass at any instant [64,65].

$$\frac{dX}{dt} = \mu X \tag{6}$$

The growth of cell is governed by hyperbolic relationship, and there is a limit to the maximum attainable cell biomass concentration. Such growth kinetics is described by a logistic equation:

$$\frac{dX}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right) \tag{7}$$

where, X is the biomass concentration (g $L^{-1}$), $X_{max}$ is the maximum biomass concentration predicted by model (g $L^{-1}$), dX/dT is the rate of biomass production (g $L^{-1}$ $h^{-1}$), $\mu$ is the specific growth rate and μmax is the maximum specific growth rate ($h^{-1}$)

The integrated form of Eq. (7) is

$$X = \frac{X_o \exp(\mu_{max} t)}{1 - \left(\frac{X_o}{X_{max}}\right)(1 - \exp(\mu_{max} t))} \tag{8}$$

The kinetic parameter $\mu_{max}$ in this equation is determined by rearranging the above equation and plotting ln(X′/1−X′) v/s t should give a straight line of slope $\mu_o$ and intercept $-\ln((X_{max}/X_o)-1)$.

where, $X_0$ is the initial biomass concentration (g $L^{-1}$), t is time (h), $\mu_0$ is specific initial specific growth rate, $X_0$ is the biomass concentration when t=0 and X′=(X/$X_{max}$).

$$\mu_0 t = \ln\left(\frac{X_{max}}{X_0} - 1\right) + \ln\left(\frac{X'}{1 - X'}\right) \tag{9}$$

3-4-2. Luedeking-Piret Equation

The kinetics of rL-asp II production was described by Luedeking-Piret equation, which states that the product formation rate depends upon both the instantaneous biomass concentration (X) and growth rate (dX/dt) in a linear fashion [66-68].

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{10}$$

where, $\alpha$ is a growth associated constant (mg $g^{-1}$) and $\beta$ is a non-growth associated constant (mg $g^{-1}h^{-1}$). The values of $\alpha$ and $\beta$ depend mainly on batch fermentation conditions.

Integrating Eq. (10),

$$P_t = P_0 + \alpha A(t) + \beta B(t) \tag{11}$$

where $P_0$ and $P_t$ are the product concentrations at initial time and at any time (at time t), respectively

$$A(t) = X_o\left[\frac{e^{\mu_o t}}{1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})} - 1\right] \tag{12}$$

$$B(t) = \frac{X_{max}}{\mu_0}\ln\left[1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})\right] \tag{13}$$

The parameters $\alpha$ and $\beta$ in Eq. (11) are determined by plotting $(P_t - P_o)/B(t)$ vs A(t)/B(t), which is a straight line with slope '$\alpha$' and intercept '$\beta$'

3-4-3. Modified Luedeking-Piret Equation

The substrate utilization kinetics is given by the following equation, which considers substrate conversion to cell mass, product and maintenance energy [65,69].

$$\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} - \frac{1}{Y_{P/S}}\frac{dP}{dt} - k_e X \tag{14}$$

where $Y_{X/S}$ and $Y_{P/S}$ are yields of cell mass and product with respect to substrate and $K_e$ is the maintenance coefficient for cells. Rearranging the substrate material balance equation

$$\frac{dS}{dt} = -\gamma\frac{dX}{dt} - \eta X \tag{15}$$

$$\text{where, } \gamma = \frac{1}{Y'_{X/S}} + \frac{\alpha}{Y'_{P/S}} \text{ and } \eta = \frac{\beta}{Y'_{P/S}} + k_e \tag{16}$$

Eq. (15) is the modified Luedeking-Piret equation for substrate utilization kinetics.

Substituting for $\mu$ from Eq. (7) and integrating gives

$$S_t = S_0 - \gamma m(t) - \eta n(t) \tag{17}$$

where, $S_0$ and $S_t$ are the substrate concentrations at initial time and at any time 't', respectively,

$$m(t) = X_o\left[\frac{e^{\mu_o t}}{1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})} - 1\right] \tag{18}$$

$$n(t) = \frac{X_{max}}{\mu_0}\ln\left[1 - \frac{X_o}{X_{max}}(1 - e^{\mu_o t})\right] \tag{19}$$

Kinetic parameters ($\gamma$, $\eta$) in Eq. (17) are determined by plotting $S_o - S_t/n(t)$ vs m(t)/n(t) which is a straight line with slope $\gamma$ and intercept $\eta$.

**4. *In Vitro* Inhibition of Polyacrylamide Formation**

The experiment was performed according to the protocol of Meena et al. [70]. The total reaction mixture consisted of 5.0 ml of 10% acrylamide solution, 2.5 ml of Tris-HCl buffer (pH 8.6) and enzyme. The reaction mixture was incubated at 45 °C for 30 min.

After incubation, 200 µl of 10% ammonium persulphate and 20 µl of tetramethylethylenediamine were added. Tubes were kept at room temperature and time of solidification was noted. The specificity of rL-asp II enzyme towards L-asparagine and acrylamide was studied according to the protocol of Chityala et al. [18].

**5. L-asparaginase Assay and Protein Estimation**

The enzymatic assay was performed according to Chityala et al. [18]. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of ammonia per minute at 37 °C. Specific activity was expressed as units per milligram of protein. Protein estimation was performed according to Bradford [71].

**6. Analytical Methods**

The growth profile of *Bacillus subtilis* WB800N/pHT43-*ans B2* was monitored by measuring the absorbance of cells at 600 nm ($OD_{600\,nm}$) with a UV-visible spectrophotometer (Cary 50, Varian, Australia) and was expressed in terms of dry cell weight (DCW) using the correlation equation $DCW=0.45\times OD_{600\,nm}$. The sucrose was estimated using a high-pressure liquid chromatograph (HPLC) (Agilent 1220 Infiniti HPLC, USA) equipped with SUPELCOGEL Ca, 300 mm×7.8 mm I.D., 9 µm particle. The acetate and acetoin were estimated using Rezex ROA-organic acid H+ (8%) column (300 mm×7.8 mm, Phenomenex, USA) linked to a guard column (50 mm×7.8 mm, Phenomenex, USA) using 0.005 N $H_2SO_4$ as mobile phase at a flow rate of 0.5 ml $min^{-1}$. CHNS analysis was performed by collecting the biomass of *Bacillus subtilis* WB800N/pHT43-*ans B2* during different experiments and dried overnight in oven at 70 °C. Then dried sample was made into fine powder and analyzed using CHNS analyser.

## RESULTS AND DISCUSSION

L-asparaginase is gaining importance due to its industrial, clinical, economic and immunological suitability. From the industrial perspective, high level of production and process economics are of major concerns. Many studies have shown that the microbial growth, metabolism and productivity is influenced by nutritional and physiological properties [72-77]. With respect to biotechnological perspective, optimization of such parameters is of central importance since a small improvement in the production titre will be crucial for a commercial success. Thus considering the above aspects, we focused on optimization of nutritional parameters to enhance the expression of rL-asp II. All the experimental samples were induced through consecutive intermittent IPTG addition. Addition of higher concentration of IPTG at single time increased the metabolic burden [78] and may have led to the formation of inclusion bodies [113]. To reduce the metabolic burden caused by IPTG [79], IPTG was added consecutively at lower concentrations. Adding lower quantities of IPTG reduces the metabolic burden [80]. In our previous studies, adding small quantities of IPTG at regular intervals of time enhanced the rL-asp II production [18]. Hence, we carried out all the experiments by adding the IPTG consecutively and intermittently.

**1. Media Engineering for the Optimized Expression of rL-asp II**

1-1. Effect of Carbon Source

As the carbon source has a major role on productivity by influencing the carbon flux, till date many investigations have been carried out for selecting the best carbon sources which maximize the L-asparaginase II production [59,70,81]. In the present study, we made an effort to screen the best carbon sources, which influence the rL-asp II expression in *Bacillus subtilis* WB800N. Based on the literature survey, we selected eleven different carbon sources affecting the rL-asp II production, as mentioned earlier within the range of 25 g $L^{-1}$. It was interesting to know that sucrose tends to show higher specific growth rate of 0.043 $h^{-1}$ and 57.9 IU/ml of rL-asp II production using *Bacillus subtilis* WB800N/pHT43-*ans B2* expression system. This findings are comparable with Jia et al. [82], where they found maximum L-asparaginase production from *Bacillus subtilis* B11-06 using sucrose as the major source of carbon. Similar results were reported by Gu et al. and Jacques et al. [42,83], who reported a substantial effect on lipopeptide production from *Bacillus subtilis* MO-01 and *Bacillus subtilis* S499 respectively using sucrose. The expression profile of different carbon sources affecting specific growth rate along with the production of over flow metabolite's such as acetate and acetoin is recorded in Fig. 1(a). It was observed that after sucrose, glucose tends to show high specific growth rate with high rL-asp II production followed by mannitol, pyruvate and sorbitol, with less acetoin and acetate production vice versa, respectively.

1-2. Effect of Nitrogen Source

In microorganisms, the nitrogen source plays a crucial role in protein expression during the transcription of carbon metabolizing gene, which depends on the source of nitrogen [84]. To understand the effect of nitrogen, we studied various organic (yeast extract, casamino acids and peptone), inorganic ($NH_4SO_4$+$NH_4Cl$, $NH_4SO_4$ and $NH_4Cl$) and amino acids (glycine and L-asparagine) as a nitrogen sources on the expression of rL-asp II. Among the tested sources, ammonium chloride showed maximum rL-asp II expression (74.1 IU/ml), with a specific growth rate of 0.0445 $h^{-1}$. These findings are comparable with previous report of Hymavathi et al. [85], who found maximum L-asparaginase production using ammonium chloride as the sole source of nitrogen with *Bacillus circulans* (MTCC8574) as an expression host. In another report the maximum amount of bio-surfactants production was reported using ammonium chloride as nitrogen source with the *Bacillus subtilis* strains [86]. When organic nitrogen sources such as yeast extract were added, the specific growth rate was very high, but the expression of rL-asp II was very low. This may be due to high production of acetate and acetoin where the metabolic flux might be diverted towards the less efficient pathways [87], which is depicted in Fig. 1(b). These results are in accordance with the previously reported findings where, when the yeast extract was used as nitrogen source, high acetoin was produced, but using ammonium chloride less acetoin was produced by *B. subtilis* SF4-3 [88]. This is the first study to show expression of novel glutaminase-free L-asp II using sucrose and $NH_4Cl$ as a carbon and nitrogen source in *Bacillus subtilis* WB800N, respectively, with less acetoin and acetate.

**2. Evaluation of Significant Medium Components Affecting rL-asp II Expression by the Plackett-Burman Experimental Design**

Plackett-Burman design is a very basic and useful statistical tool for screening of significant media components [55]. In the current

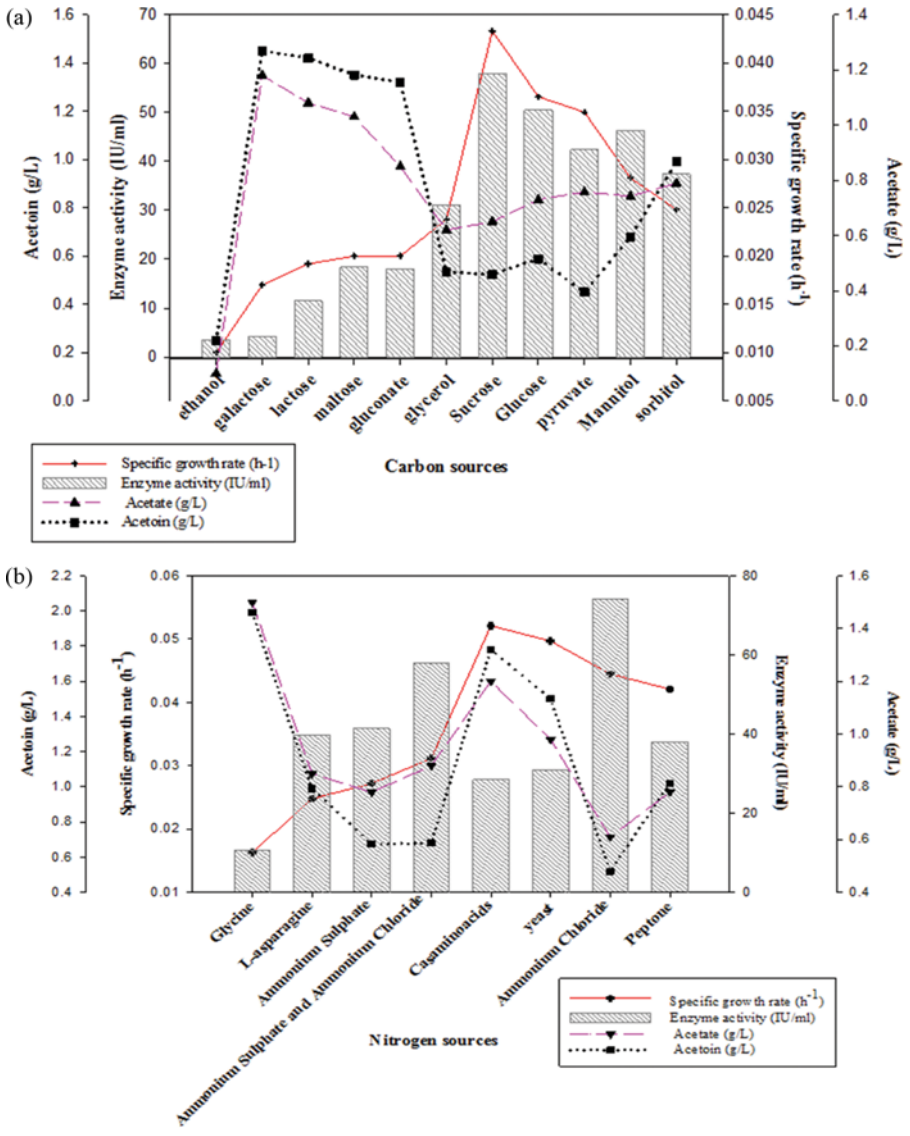


**Fig. 1. (a) Effect of carbon source on the expression of rL-asp II. (b) Effect of nitrogen source on the expression of rL-asp II.**

**Table 3. Plackett-Burman design matrix for eight variables with coded values along with the observed and predicted rL-asp II expression**

| Run order | Experimental values | | | | | | | | Enzyme activity (IU/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | [a]Observed values | Predicted values |
| 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 28.72±0.99 | 30.7221 |
| 2 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 24.928±0.56 | 24.144 |
| 3 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 3.982±0.265 | 4.864 |
| 4 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | 29.876±0.125 | 29.53 |
| 5 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | 15.192±0.85 | 15.97 |
| 6 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | 2.152±0.872 | 1.27 |
| 7 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 11.892±0.452 | 12.22 |
| 8 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | 20.072±0.452 | 18.07 |
| 9 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 13.891±0.24 | 15.89 |
| 10 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 21.161±0.124 | 20.377 |
| 11 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | 11.572±0.356 | 11.23 |
| 12 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 33.0194±0.124 | 32.138 |

[a]The observed values of rL-asp II activity, were the mean values of duplicates with standard deviation (mean±SD)

**Table 4. Statistical analysis of Plackett-Burman design showing effect, coefficient values, t and P-value for each variable**

| Term | Symbol code | Effect | Coef | T | P |
|---|---|---|---|---|---|
| Constant | | | 18.038 | 26.61 | 0.000[a] |
| Sucrose (g/L) | $X_1$ | 4.6 | 2.3 | 3.39 | 0.043[a] |
| $NH_4Cl$ (g/L) | $X_2$ | −12.837 | −6.418 | −9.47 | 0.002[a] |
| $Na_2SO_4$ (g/L) | $X_3$ | −3.845 | −1.922 | −2.84 | 0.066[b] |
| $K_2HPO_4$ (g/L) | $X_4$ | 2.541 | 1.27 | 1.87 | 0.158[b] |
| $NaH_2PO_4$ (g/L) | $X_5$ | −10.592 | −5.296 | −7.81 | 0.004[a] |
| $MgSO_4$ (g/L) | $X_6$ | −5.895 | −2.948 | −4.35 | 0.022[a] |
| Tryptophan (g/L) | $X_7$ | 0.127 | 0.063 | 0.09 | 0.931[b] |
| TES (ml/L) | $X_8$ | −2.298 | −1.149 | −1.7 | 0.189[b] |

[a]Significant
[b]Nonsignificant at P>0.05
R-Sq=98.49% R-Sq (pred)=75.83% R-Sq (adj)=94.46%

investigation, with the aim of maximizing the expression of rL-asp II, eight variables were selected and their effects were studied using predetermined matrix using MINI Tab® 16 statistical software. The design matrix selected for the screening of significant variables for rL-asp II expression and the corresponding responses are represented in Table 3. The experiments based on Plackett-Burman design showed a wide variation from 2.15 to 33.019 IU/ml of enzyme activity. This variation reflects the significance of medium components optimization to achieve higher productivity. The adequacy of the model was calculated and the variables with statistical significance were screened via student's t-test (Table 4).

Generally, factors with higher t value and lesser p value are considered as significant model term. Factors evidencing P-values of less than 0.05 were considered to have significant effects on the response, and were therefore selected for further optimization studies. In the present study, sucrose, $NH_4Cl$, $NaH_2PO_4$ and $MgSO_4 \cdot 7H_2O$ emerged as the significant variables affecting the production of rL-asp II. Among these variables $NH_4Cl$, $NaH_2PO_4$, $MgSO_4 \cdot 7H_2O$ showed negative effect, while sucrose was found to show a positive influence. A positive symbol signifies that higher level of the variable has higher response, whereas the negative sign indicates that lower level of variable is responsible for higher response [89]. While the rest of the parameters showed no significant influence. Hence, they were maintained at their middle level (center point), as these variables are also crucial for growth of the organism. Thus neglecting the terms that were insignificant, the model equation for rL-asp II enzyme activity can be written as:

$$Y_{rL\text{-}asp\ II} = 18.038 - 6.418X_2 - 5.293X_5 - 2.948X_6 + 2.3X_1 \quad (20)$$

where $X_1$, $X_2$, $X_5$ and $X_6$ are sucrose, $NH_4Cl$, $NaH_2PO_4$ and $MgSO_4$, respectively.

The Pareto chart (Fig. 2) is a convenient method of representing the effect of variables on responses in Placket-Burman design. It indicates the ranking of the variables based on the absolute values of standardized effect. The reference line (3.18 in Fig. 2) indicates that effects were significant with $\alpha$ value of 0.05. The standardized effects were the t statistics shown in Fig. 2. It is quite evident from

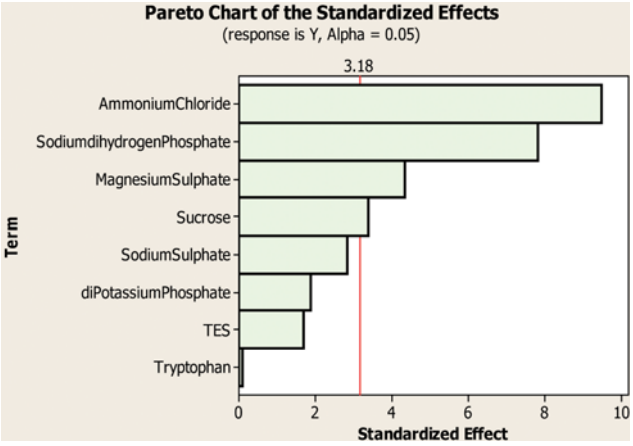


**Fig. 2. Pareto chart of standardized effects of the factors on rL-asp II expression. Media components denoted in g/L.**

**Table 5. A 24 full-factorial central composite design matrix of four variables in coded units with experimental and predicted values of rL-asp II expression**

| Run order | Coded levels | | | | Enzyme activity (IU/ml) | |
|---|---|---|---|---|---|---|
| | X1 | X2 | X5 | X6 | Observed values[a] | Predicted values |
| 1 | −1 | −1 | −1 | −1 | 105.8797±1.79 | 108.508 |
| 2 | 1 | −1 | −1 | −1 | 99.3891±1.68 | 101.627 |
| 3 | −1 | 1 | −1 | −1 | 196.782±2.58 | 187.532 |
| 4 | 1 | 1 | −1 | −1 | 108.7012±3.87 | 112.451 |
| 5 | −1 | −1 | 1 | −1 | 111.6224±1.99 | 127.259 |
| 6 | 1 | −1 | 1 | −1 | 138.2836±1.79 | 154.431 |
| 7 | −1 | 1 | 1 | −1 | 182.7376±3.56 | 174.352 |
| 8 | 1 | 1 | 1 | −1 | 146.9928±0.61 | 133.324 |
| 9 | −1 | −1 | −1 | 1 | 115.6162±1.77 | 123.771 |
| 10 | 1 | −1 | −1 | 1 | 141.51±1.96 | 149.362 |
| 11 | −1 | 1 | −1 | 1 | 381.425±3.64 | 364.745 |
| 12 | 1 | 1 | −1 | 1 | 343.286±4.00 | 322.136 |
| 13 | −1 | −1 | 1 | 1 | 153.8898±1.46 | 149.608 |
| 14 | 1 | −1 | 1 | 1 | 205.5154±1.92 | 209.252 |
| 15 | −1 | 1 | 1 | 1 | 366.4016±3.50 | 358.65 |
| 16 | 1 | 1 | 1 | 1 | 353.2558±3.58 | 350.095 |
| 17 | 0 | 0 | 0 | 0 | 320.3629±3.55 | 323.025 |
| 18 | 0 | 0 | 0 | 0 | 318.574±3.00 | 323.025 |
| 19 | 0 | 0 | 0 | 0 | 320.3731±4.17 | 323.025 |
| 20 | 0 | 0 | 0 | 0 | 308.6056±4.00 | 323.025 |
| 21 | −2 | 0 | 0 | 0 | 205.15±2.55 | 212.092 |
| 22 | 2 | 0 | 0 | 0 | 197.5507±2.72 | 196.655 |
| 23 | 0 | −2 | 0 | 0 | 128.45±0.64 | 99.371 |
| 24 | 0 | 2 | 0 | 0 | 284.1124±3.55 | 319.238 |
| 25 | 0 | 0 | −2 | 0 | 195.21±2.92 | 203.416 |
| 26 | 0 | 0 | 2 | 0 | 252.286±3.12 | 250.126 |
| 27 | 0 | 0 | 0 | −2 | 29.896±2.84 | 22.325 |
| 28 | 0 | 0 | 0 | 2 | 240.7421±3.11 | 254.359 |
| 29 | 0 | 0 | 0 | 0 | 320.3731±4.11 | 322.279 |
| 30 | 0 | 0 | 0 | 0 | 348.372±1.28 | 322.279 |

the figure that variables such as sucrose, $NH_4Cl$, $NaH_2PO_4$ and $MgSO_4$ were influencing the production of rL-asp II significantly. The Plackett-Burman design allowed us to define a new medium composition for the production of rL-asp II [83]. Similar results were observed by Gu et al. for the production of a novel lipopeptide by *Bacillus subtilis* MO-01 using sucrose and ammonium chloride along with zinc sulfate [42]. Kim et al. [90] also observed that screening of media components using combined statistical designs enhanced the production of extracellular proteolytic enzyme by *Bacillus subtilis* FBL-1.

**3. RSM Optimization for rL-asp II Expression**

The CCD is a full factorial design, composed of a cube part which allows determination of main and interaction effects and a star design ($\alpha$) for quantifying main and quadratic effects [91]. The experiments were performed as mentioned in the materials and methods section. The design matrix and the corresponding responses of CCD experiments are shown in Table 5, along with the mean predicted values. The results were analyzed using ANOVA (Table 6).

**Table 6. Analysis of variance (ANOVA) for quadratic model**

| Source | DF | SS | MS | F-value | P-value |
|---|---|---|---|---|---|
| Model | 14 | 278218 | 19872.7 | 53.51 | <0.000 |
| Residual (error) | 14 | 5199 | 371.4 | - | - |
| Lack-of-fit | 10 | 4712 | 471.2 | 3.87 | 0.102 |
| Pure error | 4 | 488 | 121.9 | - | - |
| Total | 29 | 283421 | | | |

$R^2$=98.17%; Adj $R^2$=88.39%; pred $R^2$=96.2%

SS, sum of squares; DF, degrees of freedom; MS, mean square

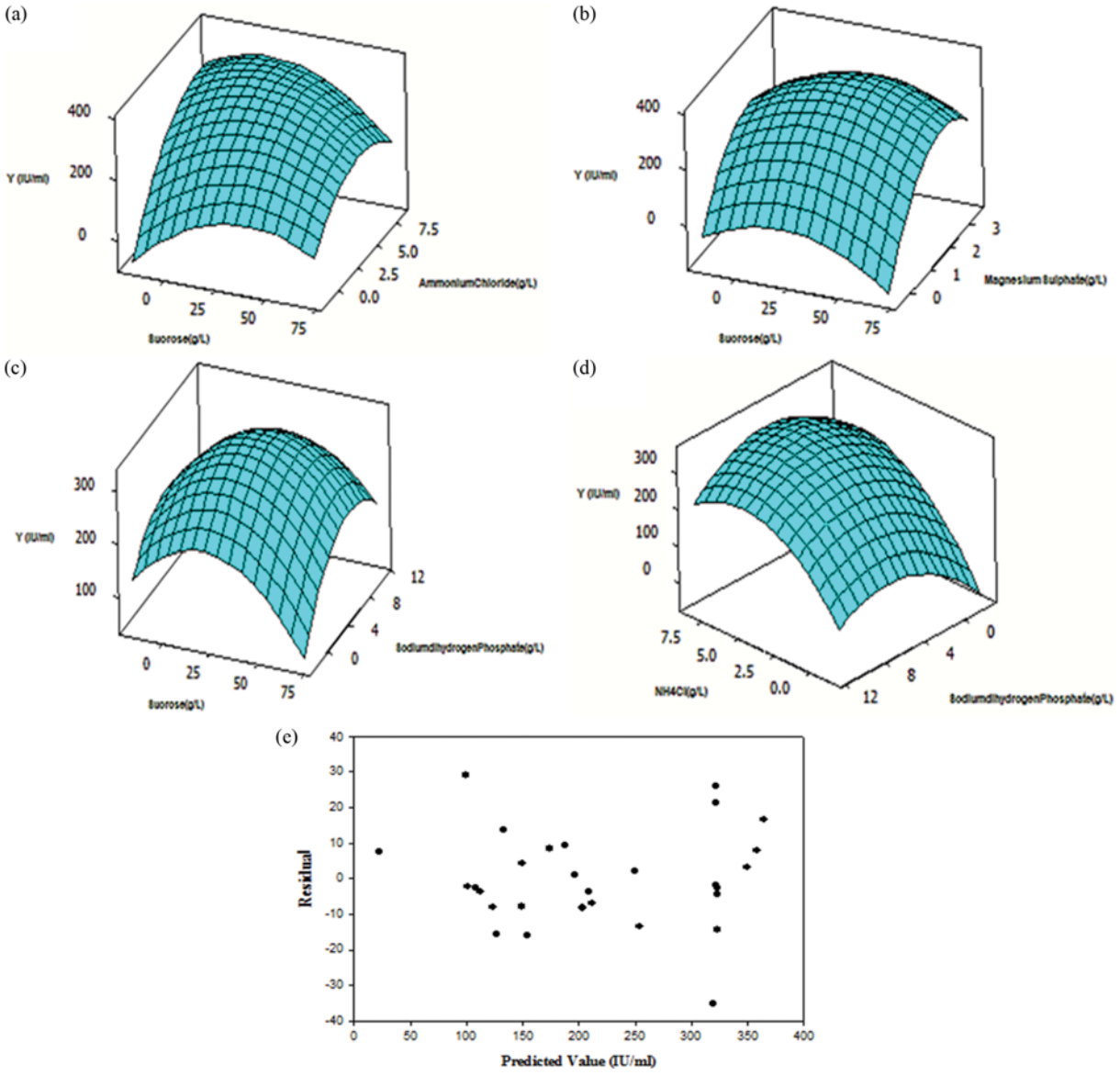


**Fig. 3. Three-dimensional response surface plot for rL-asp II expression showing the interactive effects of (a) sucrose and $NH_4Cl$ (b) sucrose and $MgSO_4$ (c) sucrose and $NaH_2PO_4$ (d) $NaH_2PO_4$ and $NH_4Cl$ (e) residuals plotted against predicted values of $Y_{rLasp\ II\ activity}$ (IU/ml) from RSM.**

According to ANOVA, the quadratic model proved to be highly significant with the Fisher F test (mean square regression: mean square residual is 53.51) with a very low probability value ($P_{model}$> 0.05). The residuals were plotted against the predicted values of $Y_{rLasp\ II\ activity}$ in Fig. 3(e). The horizontal band indicates no unusual behavior or abnormality [42,92], which explains the adequacy of the model. The goodness of fit for the model was determined through coefficient of determination value, and it was evident that the model is highly significant with $R^2$ of 0.9817. This shows that the model is capable of explaining 98.17% of the variation in response. Also, the lack of fit of the model has shown an insignificant value (p>0.05) with F value of 3.87. This represents that the lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the model [93]. By applying the multiple regression analysis on the experimental data, the following second order polynomial was given by:

$$\begin{aligned} Y_{rL\text{-}asp\ II\ activity} = {} & 322.652 - 3.859X_1 + 54.967X_2 + 11.678X_5 + 58.008X_6 \\ & - 29.476X_1^2 - 28.244X_2^2 - 23.877X_5^2 - 45.984X_6^2 \\ & - 17.05X_1X_2 + 8.513X_1X_5 + 8.118X_1X_6 \\ & - 7.983X_2X_5 + 40.487X_2X_6 + 1.771X_5X_6 \end{aligned} \quad (21)$$

The student's t distribution and the corresponding P values, along with the parameter estimate are shown in Table 7. It is evident from the Table 7 that all linear and square terms except sucrose have shown a significant effect on rL-asp II expression, whereas the interaction terms between sucrose and $NH_4Cl$, $NH_4Cl$ and $MgSO_4$ were highly significant on the production. Similar observations were reported by Liu et al. [94], where including the interaction effects of parameters in the model showed significant improvement in the expression of HAS/lL 1Ra in *Pichia pastoris*. To understand the effect of variables and interaction among them on rL-asp II expression better, three-dimensional response surface plots were constructed by MINITAB® Release 16.1.1, PA, USA. In Fig. 3(a), there is a steep increase in rL-asp II expression with increase in ammonium chloride and sucrose concentration, but the rL-asp II expression de- creased at higher concentration of sucrose, which may be due to the substrate inhibition [95].

**Table 7. Model coefficient estimated by multiple linear regressions**

| Model term | Coef | SE Coef | Computed t-value | *P*-value |
|---|---|---|---|---|
| Constant | 322.652 | 7.965 | 40.507 | 0.000 |
| $X_1$ | −3.859 | 3.934 | −0.981 | 0.343 |
| $X_2$ | 54.967 | 3.934 | 13.973 | 0.000 |
| $X_5$ | 11.678 | 3.934 | 2.969 | 0.010 |
| $X_6$ | 58.008 | 3.934 | 14.746 | 0.000 |
| $X_1^2$ | −29.476 | 3.680 | −8.011 | 0.000 |
| $X_2^2$ | −28.244 | 3.680 | −7.676 | 0.000 |
| $X_5^2$ | −23.877 | 3.680 | −6.489 | 0.000 |
| $X_6^2$ | −45.984 | 3.680 | −12.497 | 0.000 |
| $X_1X_2$ | −17.05 | 4.818 | −3.539 | 0.003 |
| $X_1X_5$ | 8.513 | 4.818 | 1.767 | 0.099 |
| $X_1X_6$ | 8.118 | 4.818 | 1.685 | 0.114 |
| $X_2X_5$ | −7.983 | 4.818 | −1.657 | 0.120 |
| $X_2X_6$ | 40.487 | 4.818 | 8.404 | 0.000 |
| $X_5X_6$ | 1.771 | 4.818 | 0.368 | 0.719 |

A similar profile was observed in Fig. 3(d), Fig. 3(b) and Fig. 3(c), with $NaH_2PO_4$ and $NH_4Cl$ (p value 0.120), sucrose and $MgSO_4$ (p value 0.114) and sucrose and $NaH_2PO_4$ (p value 0.099), respectively. The experimental data were fitted into the aforementioned Eq. (21), and the optimum levels of each variable were determined to be as follows: sucrose 17.0455 (g/L), $NH_4Cl$ 8.5 (g/L), $NaH_2PO_4$ 4.146 (g/L), $MgSO_4$ 2.974 (g/L) with an over-all yield of 389.56 IU/ml of rL-asp II production. An overall yield of 5.25-fold increase in rL-asp II production was achieved using the Plackett-Burman design followed by the central composite experimental design technique compared to un-optimized medium. Similar reports of enhanced production of L-asparaginase II enzyme were noted while optimizing the culture conditions with different strains [74,96-101]. However, very few studies deal with statistical optimization of the process for recombinant L-asp II expression in *Bacillus subtilis* [82]. To our best knowledge, there are no reports available on optimization of media for the production of rL-asp II production using sucrose and ammonium chloride as a carbon and nitrogen sources from *Bacillus subtilis* WB800N.

**4. Hybrid Model of Statistical Analysis Using Artificial Neural Network Linked Genetic Algorithm (ANN-GA)**

4-1. Artificial Neural Network

In this study, we used the feed forward back propagation algorithm. The input neuron represents sucrose, $NH_4Cl$, $MgSO_4$ and $NaH_2PO_4$. While the output layer represents rL-asp II expression. The training was done for 1000 epochs. The optimal results were found at 9 epochs with 4 inputs, 3 hidden layers and 1 output layer (Fig. 4(a)). The MSE and determination coefficient ($R^2$) for training, validation and test are shown in Table 8. The parity plot of experimental output versus prediction output is shown in Fig. 4(b). The model $R^2$ and adjusted $R^2$ were found to be 0.971 and 0.989, respectively.

4-2. Genetic Algorithm Based Optimization

Once the Neural network was trained, GA was used to further optimize the input spaces for maximizing rL-asp II expression. The values of GA specific parameters used in the optimization technique were as follows: population size=20, cross over probability=0.8, mutation probability=0.01, no. of generations=100. To achieve optimum global solution, GA was repeated several times with different initial values. The maximum predicted value of 546.46 IU/ml rL-asp II was achieved by maintaining the parameters at sucrose 36.12 (g/L), $NH_4Cl$ 5.99 (g/L), $NaH_2PO_4$ 1.19 (g/L), $MgSO_4$ 1.58 (g/L), after 100 iterations and the probability of optimal variable solution was found (Fig. 4(c)).

**5. Verification of Model from RSM and ANN Linked GA**

To validate the above proposed experimental models, validation experiments were performed for RSM and ANN linked GA. The experiments were carried out at optimum conditions predicted by RSM, then the experimental and the predicted output data from the model were evaluated. It was found that the experimental output data (rL-asp II activity) was 389.56±1.89 IU/ml, whereas the predicted value from the polynomial model was 430.602 IU/ml. The verification of the model revealed a high degree of accuracy,

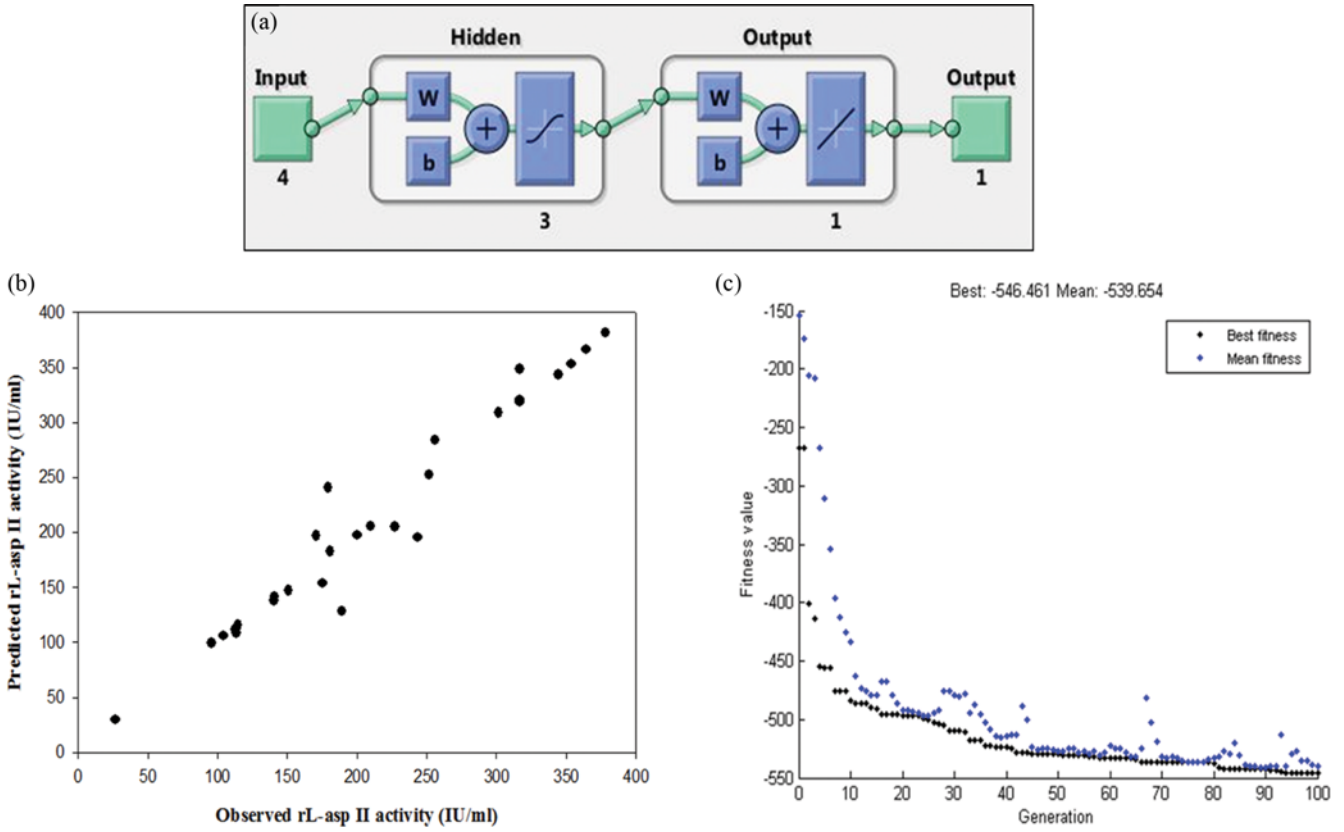


**Fig. 4. (a) Artificial Neural Network architecture showing the input, hidden and output layer, (b) parity plot showing the goodness-of-fit for the ANN model (* $R^2$=97.1% and $Radj^2$=98.9%), (c) representative plots generated from the optimization by GA using MATLAB (2011b) best and average fitness values with successive generations showed gradual convergence to the optimum value for rL-asp II expression.**

**Table 8. Artificial Neural Network Architecture MSE and $R^2$ prediction of rL-asp II expression**

| | Samples | MSE | $R^2$ |
|---|---|---|---|
| Training | 20 | 0.64 | 0.99 |
| Validation | 5 | 0.81 | 0.89 |
| Testing | 5 | 0.58 | 0.97 |

more than 90.46%, which is an evidence for the good model validation under the investigated conditions. The validation experiment for ANN linked GA showed that 525.98±2.04 IU/ml, which is in close agreement with the hybrid ANN-GA output data of 546.46 IU/ml. The accuracy of the model was found to be 96.25%. The production of rL-asp II from WB800N is higher than the L-asparaginase produced from *B. subtilis* WB600 through a combined strategy during fed batch conditions (407.6 IU/mL) [102]. It was observed that ANN-GA was more efficient than that of CCD with an $R^2_{ANN\text{-}GA}$ 96.26%>$R^2_{CCD}$ 90.46% (Table 9). The results are in accordance with the recent research articles where many scientists reported that ANN-GA was found to be more precise than RSM model [101,103-107]. The maximum biomass was found to be 7.52 DCW/L with production of rL-asp II 525.98 IU/ml during batch bioreactor. An overall increase of 7.098-fold in rL-asp II expression was observed compared to un-optimized with ANN-GA. In this study, the rL-asp II expression was observed to be higher than the reported values from different strains such as, *Bacillus subtilis* WB600 407.6 IU/ml [102], *Aspergillus terreus* MTCC1782 36.97 IU/ml [108], *Pectobacterium carotovorum* MTCC1428 14.53 IU/ml [109], *Pichia pastoris* (PichiaPink) 2.5 IU/ml [15], *Bacillus aryabhattai* ITBHU02 6.35 IU/mg [101] and *Streptomyces ginsengisoli* 3.23 μmol/mL/min [110]. This shows the efficacy of the WB800N strain.

**Table 9. RSM and ANN linked GA for modelling and optimization of rL-asp II expression**

| Variable | Optimum concentration (g/L) | | | | rL-asp II activity (IU/ml) | | $R^2$ value |
|---|---|---|---|---|---|---|---|
| | X1 | X2 | X5 | X6 | Predicted | Observed | |
| RSM model | 17.0455 | 8.5 | 4.146 | 2.974 | 430.602 | 389.56 | 0.9046 |
| ANN linked GA | 36.12 | 5.99 | 1.19 | 1.58 | 546.46 | 525.98 | 0.9626 |

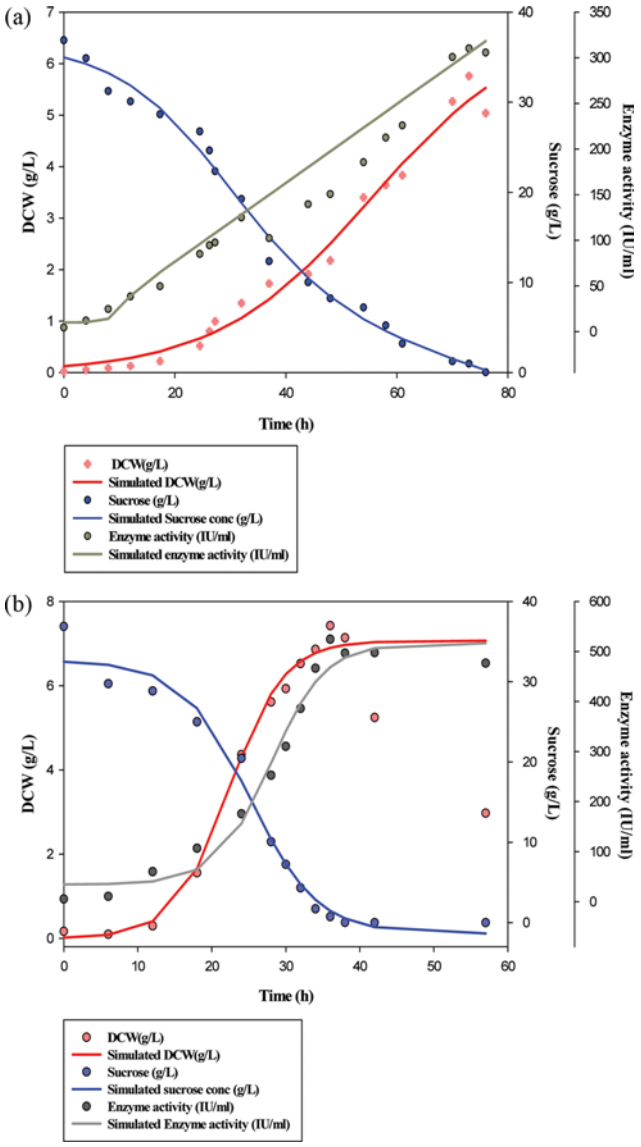


**Fig. 5. Experimental and model simulated growth kinetics, production kinetics and substrate consumption kinetics. (a) Un-optimized bioreactor during rL-asp II production. (b) Optimized bioreactor rL-asp II production.**

## 6. Unstructured Bio-kinetic Modeling for Prediction of rL-asp II Fermentation

The experimental and predicted profiles for the production of rL-asp II in batch bioreactor under un-controlled and controlled conditions are shown in Fig. 5(a) and 5(b), respectively. It was observed that under uncontrolled conditions the rL-asp II expression in batch fermentation increased up to 73 h (310.20 IU/ml), after which it declined. While under controlled conditions the rL-asp II expression enhanced up to 36 h (525.98 IU/ml) and then declined gradually. The kinetic parameters involved in the process were estimated using different models mentioned in Eqs. (8), (11) and (17). The fitting of experimental data with the bio-kinetic models, non-linear regression using the least-square method was employed to predict the model simulated data using Microsoft Excel Solver 2003. The estimated kinetic parameters from these models are mentioned in Table 10. The coefficients of determination ($R^2$) values obtained by fitting various models to experimental data were found to be highly significant. The Luedeking-Piret model for rL-asp II expression suggested that the rL-asp II expression is mixed growth associated in uncontrolled bioreactor, while under controlled conditions it showed growth associated production (Table 10) [89]. The modified Luedeking-Piret model for substrate consumption profile of model simulated and predicted is shown in Fig. 5(a) and 5(b). The maximum biomass, rL-asp II expression, specific growth rate, total protein expression, acetate and acetoin was found to be 7.52 g/L, 525.98 IU/ml, 0.21 $h^{-1}$, 8.83 g/L, 0.79 g/L and 0.645 g/L, respectively.

During the sequential optimization of media and physical parameters from LB media to ANN through RSM, the expression profile of rL-asp II was enhanced considerably (Fig. 6). The CHNS analysis clearly showed that the % composition of C, H, N, S (Fig. 6) distributed in the biomass in different experiments showed that the metabolic flux is directed towards the rL-asp II expression and other maintenance factors rather than enhancing biomass [87]. The acetate and acetoin was maintained at very low levels in ANN optimized experiment. The total protein production was also enhanced when the parameters were optimized. This clearly shows that optimization of physical and media components for high yield expression of rL-asp II is very essential in the biotechnological perspective.

## 7. Effect of rL-asp II on *In Vitro* Polyacrylamide Formation

To check the effect of polyacrylamide formation, experiments were performed to study the rate of solidification of the samples. All the experiments were performed by taking 10% of 5 ml acrylamide solution. The enzyme concentration of 0, 15, 30, 60 and 90 IU of crude and purified rL-asp II was taken to check the solidification conditions. The immediate solidification was observed in the tubes in which rL-asp II was not added, whereas delayed solidification or no solidification was observed in tubes added with rL-asp II enzyme (Table 11). The purified rL-asp II delayed the solidification of acrylamide compared to the crude rL-asp II. It can be observed that the period of solidification was delayed with increase in purified rL-asp II concentration above 60 min. The findings of

**Table 10. Parameters estimated by logistic and Leudeking-Pirate model equation**

| Model | Bioreactor | Parameters | $R^2$ |
|---|---|---|---|
| Logistic model | | $X_0$ (g/L)=0.033, $X_{max}$ (g/L)=5.79, $\mu$ ($h^{-1}$)=0.0802 | 0.983 |
| LP model | Un-optimized bioreactor | $\alpha$ (IU/ml)=0.0916, $\beta$ (IU/ml·h)=0.0161 | 0.941 |
| Logistic model | | $X_0$ (g/L)=0.046, $X_{max}$ (g/L)=7.52, $\mu$ ($h^{-1}$)=0.2190 | 0.99 |
| LP model | Optimized bioreactor | $\alpha$ (IU/ml)=52.511, $\beta$ (IU/ml·h)=0.86 | 0.945 |

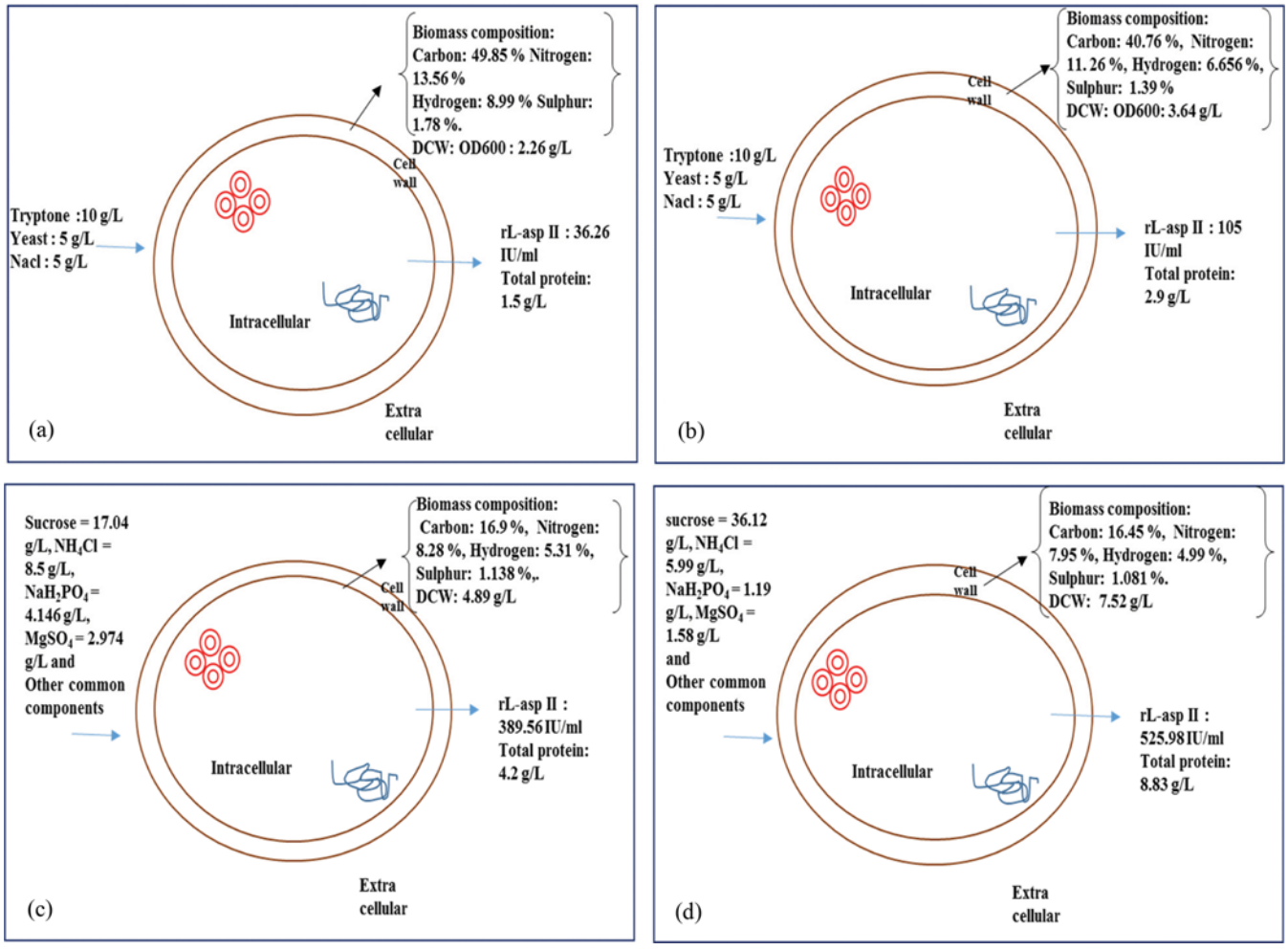


**Fig. 6. Sequential high yield expression profile of rL-asp II production by *Bacillus subtilis* WB800N in different media composition. (a) LB media with un-optimized physical factors, (b) LB media with optimized physical factors [18], (c) RSM optimized media, (d) ANN-GA optimized media.**

**Table 11. Effect of rL-asp II on *in vitro* polyacrylamide formation**

| Enzyme | Amount of enzyme (mL) | Time of solidification (min) |
|---|---|---|
| Control | - | Immediately |
| Crude extract | 0.5 | 2.5 |
| | 1.0 | 8 |
| | 2.0 | 15 |
| | 3.0 | 30 |
| Purified rL-asp II | 0.5 | 12 |
| | 1.0 | 28 |
| | 2.0 | 60 (no complete solidification) |
| | 3.0 | - |

this experiments are similar to the reported results of Meena et al. and Mahajan et al. [70,100], which shows that the rL-asp II produced from *Bacillus subtilis* WB800N is effective in inhibiting polyacrylamide formation. The acrylamide consists of two functional groups: an amide group and the vinylic carbon-carbon double bond [111,112]. The relative specific activity studies showed that rL-asp II has activity towards acrylamide but it is more specific towards L-asparagine than acrylamide [18].

## CONCLUSION

*Bacillus subtilis* is a well-known organism for production of numerous heterologous proteins and enzymes. However, the major bottleneck is the production of overflow metabolites such as acetate and acetoin, which hinders the expression of rL-asp II. To minimize the production of such overflow metabolites and to enhance the rL-asp II expression, we tried to optimize the media components by employing various statistical approaches. Optimization plays a crucial role in enhancing the expression of rL-asp II. It is very essential for optimized expression of rL-asp II, thus leading to cut down the cost of rL-asp II production. We tried to screen various carbon and nitrogen sources. The essential media components were screened using Plackett-Burman design. Further, the screened significant components were prioritized using central composite

design and ANN-GA. It was observed that ANN-GA was more efficient than that of CCD with an $R^2_{ANN\text{-}GA}$ 96.26%>$R^2_{CCD}$ 90.46%. The maximum biomass was found to be 7.52 g/L with production of rL-asp II 525.98 IU/ml during batch bioreactor. The unstructured bio-kinetic models fitted into the experimental data predicted accurately with $R^2$ of more than >0.94. The maximum predicted biomass and specific growth rate was found to be 7.52 g/L and 0.219 $h^{-1}$ respectively. This is the first study to show expression of recombinant glutaminase free L-asp II using sucrose and $NH_4Cl$ as a carbon and nitrogen source. Respectively. with less production of acetoin and acetate, which can be applied at large scale.

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## CONFLICT OF INTEREST

All authors of the paper have read and agreed to submission to your journal. The authors declare that they have no conflict of interest. This paper is not published elsewhere and it is not been submitted for publication elsewhere.

## ETHICAL APPROVAL

This article does not contain any studies with human participants or animals.

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