RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Industrial effluent as a substrate for glutaminase free L-asparaginase production from *Pseudomonas plecoglossicida* strain RS1; media optimization, enzyme purification and its characterization

Ganeshan Shakambari [a], Babu M Sumi[a], Balasubramaniem Ashokkumar [b], Peramachi Palanivelu [a] and Perumal Varalakshmi [a]*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Glutaminase free L-asparaginase is a vital enzyme because of its anti cancer potential. A potent bacterium isolated from marine environment, producing glutaminase free L-asparaginase using M-9 medium with L-asparagine was identified as *Pseudomonas plecoglossicida* RS1 by 16S rRNA gene sequencing. Statistical modeling was employed to optimize the medium using sugar cane industry effluent as a sole substrate for L-asparaginase production. The enzyme activity of L-asparaginase was increased with M-9 medium containing 0.8% effluent (3.25 ± 0.12 IU/mL) than M-9 medium containing 0.3% L-asparagine (0.73 ± 0.08IU/mL). The apparent K_m and V_{max} of the purified L-asparaginase was 2.25 ± 0.61 mM and 8.9 ± 0.81 IU/mL/min respectively and the optimal activity of L-asparaginase was at pH 8.5 and 55ºC. This study highlights use of industrial effluent as an alternate to L-asparagine for the production of L-asparaginase and to improve the cost effectiveness of this enzyme.

Introduction

L-asparaginase (EC 3.5.1.1) has been used as therapeutic remedy for acute lymphoblastic leukemia (ALL) wherein lymphoblasts are auxotrophic for L-asparagine, and the enzyme diminishes the supply of exogenous asparagine, thus forcing the tumor cells to apoptosis [1,2]. The antineoplastic activity of L-asparaginase have been explored from microbial sources such as *Escherichia coli* [3,4], *Proteus vulgaris* [5], *Serratia marcessans* [6,7], *Bacillus* sp. [8], *Pseudomonas aeroginosa* [9], *Streptomyces* sp. [10,11], *Aspergillus terreus*[12], and *Erwinia carotovora* [13].

The substrate specificity of L-asparaginase is characterized for both L-asparagine and L-glutamine where L-glutamine differs from L-asparagine by a single methyl group, as a consequence L-asparaginase treatment diminishes the L-glutamine availability along with L-asparagine concentration, causing side effects like leucopenia, acute pancreatitis, hyperglycemia and neurological seizures in ALL patients [14, 15, 16]. Hence glutaminase free L-asparaginase from microbial sources, with least cross reactivity, is a great matter of interest for the researchers, but it has not met an appreciable success. In addition, several L-asparaginases with potential catalytic properties were unsuccessful in the preclinical trials, due to their therapeutic ineffectiveness [17]. Therefore finding of glutaminase free L-asparaginase activity from a novel source is an imperative need to develop a targeted therapy which may be ameliorating the best candidate for treatment of ALL.

The fermentation process for the enhanced production of L-asparaginase was accomplished by supplementation of variety of carbon, and nitrogen sources to the media and this process may also be regulated by environmental and nutritional factors in gram negative bacteria [18]. In this perspective, it is essential to evaluate the microbial system that requires environmental friendly nutritional requirements for the growth and biocatalyst production. In recent years, many agro and industrial wastes are being used as an alternate substrate for the production of proteins as enzymes [19] and lipids etc [20]. Selection of the wastes and its parameters including cost and availability are also very important for host enzyme production thus, in this study we have utilized the sugar cane industry effluent as a substrate for L-asparaginase production since it is available in large quantity and can be obtained very easily. It is also noteworthy to mention, the statistically designed experiments like Plackett Burman Design (PBD) and Central Composite Design (CCD), would effectively embark upon the problem that involves upstream and downstream processing thus reducing the inaccuracy of interactions between nutrient parameters, and also that would aid to achieve enhanced biocatalyst production economically [21, 22]. Hence, the present study was performed to attain the improved production of L-asparaginase using industrial effluent as an alternate substrate to L-asparagine by bioprocess methodologies such as PBD and CCD. Purification and characterization of L-asparaginase was also performed. To the best of our knowledge, this is the first report highlighting sugar cane industry effluent was used as an alternate substrate to L-asparagine for the production of L-asparaginase.

Materials and Methods

Chemicals

Zobelle marine broth, Nutrient broth, D-Glucose, Na_2HPO_4, KH_2PO_4, $CaCl_2$.$2H_2O$, $NaNO_3$, sarkosyl, sodium dodecyl sulfate, Tris base were purchased from Himedia, (India). L-asparagine, L-glutamine, phenol red, DEAE sepharose, Readymix Taq Polymerase Kit, GenElute kit were obtained from Sigma Aldrich (USA). Nesslers reagent was purchased from SD Fine Chemicals (India). Ammonium sulphate was purchased from MERCK (India).

Bacterial culture and preliminary screening of L-asparaginase

Marine isolates from Gulf of Mannar near Rameswaram, India were screened for production of L-asparaginase, and the isolate positive for L-asparaginase was further identified based on its 16S rDNA sequence (SciGenome, India). Screening for L-asparaginase by plate assay method was done using M-9 medium consisted of D-Glucose- 3 g, Na_2HPO_4- 6 g , KH_2PO_4- 3 g, $MgSO_4$- 0.5 g, NaCl- 0.5 g, L-asparagine-3 g and 0.001% phenol red indicator. [22]

Quantitative assay for L-asparaginase and L-glutaminase activity

The quantitative assay was performed for the estimation of L-asparaginase and L-glutaminase activity. The 50 mL of M-9 medium consisted of the amino acids L-asparagine / L-glutamine with 2% (v/v) inoculum (*Pseudomonas plecoglossicida* RS1) were incubated at 37°C for 48 h. The cell free supernatant obtained by centrifugation at 8000 rpm for 10 min was used as source of crude enzyme to quantify the L-asparaginase / L-glutaminase activity by direct nesslerization, where the enzyme hydrolyzed product ammonia is detected and quantified based on the standard curve obtained using ammonium sulphate as the standard.[3]

One unit of enzyme activity is defined as the amount of enzyme that liberates 1 µmol of ammonia per minute at pH 8.6 and 37°C.

The total protein contents of the samples were determined according to the method by Folin Lowry method using bovine serum albumin as standard. Specific activity of the enzyme is given by enzyme activity per milligram of protein, (IU/mg protein)

Analysis for the effect of pH, temperature and substrate for L-asparaginase production by *Pseudomonas plecoglossicida* RS1

The effect of different pH (6 to 8) of the medium and incubation temperature (20°C, 37°C and 55°C) for L-asparaginase production were evaluated. Effluent from sugar cane industry was used for enhancing the production of L-asparaginase in M-9 medium that was free of L-asparagine and M-9 basal medium without any nitrogen source was used as a control. Protein concentrations were measured by Lowry's method. Reducing sugar content was estimated by DNSA method[23] where, diluted effluent (3 mL) was added to 3 mL of dinitrosalicylic acid reagent and boiled for 10 min, cooled and absorbance was read at 550 nm. The Biochemical Oxygen Demand (BOD) of the effluent was also estimated as per the stipulations of IS: 3025 (Part 44) – Reaffirmed 2003. Different concentrations of effluent (0.1% to 2%) was added to M-9 medium (50 mL) and each flask was provided with an equal 2% inoculum grown overnight ($2x10^5$ cfu/mL). The flasks were incubated at static conditions at 37°C for 48 h and the specific activity of enzyme was quantified. The study was carried out in three independent triplicate analyses.

Media optimization for the production of L-asparaginase by *Pseudomonas plecoglossicida* RS1 using Plackett Burman Design

The Plackett Burman design [24] (PBD) was used to identify the important media components to enhance the L-asparaginase production using the software Design Expert version 9.0.7. In this design, the seven crucial components of media like glucose, Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, effluent and pH were selected as the parameters for the study and the responses are, enzyme activity and specific activity of L-asparaginase. Each variable was represented as high and low levels (Supplementary table III) as described by Plackett Burman design and a total of twelve experiments were carried out. All experiments were carried out in duplicates, and the mean value of L-asparaginase enzyme units and specific activity was taken as responses (Y). The process of optimization is defined in a first order polynomial model as follows:

$$Y = \beta o + \Sigma \beta_i X_i \quad (i = 1, 2, 3\text{------}k) \qquad \text{.....(1)}$$

Where: Y – Response, βo – model intercept, β_i– linear coefficient X_i - level of independent variables.

Based on the significant influence in the response, the variables were further selected for CCD.

Central Composite Design

To optimize the levels and analyze the combined effect of screened media components that were significant for the production of L-asparaginase, the CCD was used .[8] According to this design, a total number of treatment combinations is $2^k + 2k + n_0$ where 'k' is the number of independent variables and n_0 the number of replications of the experiments at the central point, accordingly, a total of twenty experimental runs for the three components selected from PBD, with six replicates at central value were performed in duplicates and the influence of each component was evaluated at five coded levels and responses were evaluated as enzyme activity and specific activity. For calculation, the variables X_i have been coded as x_i .

For predicting the optimum levels the quadratic equation was expressed as follows:

$$Y = \beta o + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j \qquad \text{.....(2)}$$

Where Y is the response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect [6].

Verification of production media

The model was validated with the optimized medium M-9 medium (500 mL) consisting of the significant variables obtained from the CCD inoculated with 2% (v/v) inoculum, incubated at 37˚C for 48 h in static condition, and the enzyme activity and

specific activity were determined. The study was carried out in triplicates and the mean value was calculated.

Purification and characterization of enzyme

All the purification steps (Ammonium sulphate precipitation, dialysis) were carried out at 4°C, while the column purification of enzyme was done at 18°C. The chromatographic purifications were analysed for protein at 280 nm (Biophotometer D30, Eppendorf, Germany).

Ammonium sulfate precipitation

The purification of crude enzyme obtained from 500 mL of optimized production media was initiated with ammonium sulfate precipitation. Powdered ammonium sulfate was added to achieve 20% saturation and increased successively to get maximum precipitation of proteins (between 60-80 % saturation). This mixture was left for 3 h followed by centrifugation at 10000 rpm for 10 min and the precipitate was preserved. The precipitated protein was resuspended in 50 mM Tris HCl buffer of pH 8.6 and further dialyzed (Dialysis membrane-110, Himedia) against the same buffer and incubated overnight in order to remove the salt impurities, and analysed for the specific activity of L-asparaginase in the respective fractions. The dialyzed protein fraction was further applied for column purification.

DEAE Sepharose ion exchange chromatography

For further purification, the dialyzed ammonium sulfate fraction was loaded on diethylaminoethyl (DEAE) sepharose column (1 cm x 50 cm) as 2% of bed volume of 30 mL. The column was pre-equilibrated with two column volume of 50 mM Tris–HCl (pH 9.6) before loading the sample, at a flow rate of 1 ml/ min. The adsorbed protein was eluted using a linear gradient of KCl (0–200 mM) in 50 mM Tris–HCl (pH 8.6). All the fractions were collected manually and assayed for protein at 280 nm and L-asparaginase activity by direct nesslerization method[3]. The results were plotted for enzyme activity and protein absorbance (double Y plot) versus the eluted fraction, using statistical software Origin Professional version 6.0 Professional. The purified fractions of L-asparaginase were pooled and dialyzed with Tris–HCl (50 mM and pH 8.6) and were stored at -20˚C which was further used to identify the molecular weight of L-asparaginase by native polyacrylamide gel electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE).

Electrophoretic analysis of purified protein

Native PAGE analysis of the purified L-asparaginase was performed on 7.5 % polyacrylamide gel in Tris glycine buffer at pH 8.8 and SDS PAGE was done by the method of Laemmli (1970)[25] with a 12 % separating acrylamide gel (pH 8.8) and a 5 % stacking gel (pH 6.8) having 0.1% SDS. Electrophoresis was carried out using Tris glycine buffer at pH 8.3 at 120V for 3 h at room temperature (25ºC). The proteins in the gel were stained with Coomassie Brilliant Blue G-250 (SDFCL, India) for 3 h and de-stained till the dye is completely removed. The molecular weight and the subunit size of L-asparaginase were determined using the standard molecular weight markers of SDS-PAGE (Bio-Rad, USA) and native PAGE (Native Mark, Novex Life Technologies, USA).

Effect of pH, temperature and effectors on activity and stability of enzyme

The optimum enzyme activity was estimated at various pHs ranging from 4 to 9.5. The pH stability studies were performed by incubating the enzyme substrate system with acetate buffer (pH 4 to 6), potassium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.5). In order to determine the pH at which the enzyme can be stored with optimum activity, the enzyme in the absence of the substrate was stored with the buffer of different pH for 24 h at 4 °C, which was determined by direct nesslerization reaction and results of enzyme activity versus pH plotted on a graph indicated the pH stability for the purified L-asparaginase. Similarly, the optimum temperature for the enzyme was evaluated by incubating the enzyme substrate system at different temperatures ranging from 0°C to 80°C and the enzyme activity was estimated. Further, the enzyme activity in presence of various effectors (Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}, ethylene diamine tetra acetic acid (EDTA), L-cystine, L-histidine, mercaptoethanol and sodium dodecyl sulphate) was calculated after 30 min exposure of the enzyme to each effectors as reported earlier [14,26,27]. The relative activity was given as the percentage ratio of the activity of enzyme incubated with the effectors and enzyme without effectors, under standard conditions used as a control.

Kinetic parameters of L-asparaginase

Purified enzyme was suspended in 50 mM Tris-HCl buffer pH 8.6 with different concentration of substrate L-asparagine ranging from 0.01 mM to 10 mM and the Michaelis constant (K_m) and maximal velocity (V_{max}) were calculated by non linear regression analysis. The reaction velocity (V) reported as (IU/mL/min) is the mean of at least three measurements that were normalized to blank, which contained no enzyme. The obtained enzyme activity (IU/mL/min) was plotted against the substrate concentration (mM) using the software Graph Pad Prism Version 6 to obtain Michaelis Menten plot.

Results and Discussion

Screening and production of L-asparaginase by marine bacteria

Fifteen bacterial colonies were isolated from marine water sample collected from Gulf of Mannar, Tamil Nadu, India and were screened for its potential for L-asparaginase activity with M-9 medium containing L-asparagine (0.3% w/v) using phenol red plate method. [22] Among the 15 isolates, bacterial strain RS1 was found to be positive for L-asparaginase by developing pink colouration and further identified as *Pseudomonas plecoglossicida* by 16S rRNA sequence analysis showing 98% sequence similarity and 99% query coverage to *Pseudomonas plecoglossicida* strain FPC951, by BLAST analysis (www.ncbi.nlm.nih.gov/) (GenBank Accession number: KJ508408) (Supplementary Fig. A). Further, the L-asparaginase produced by *Pseudomonas plecoglossicida* RS1 showed enzyme activity 0.87 ± 0.2 IU/mL /min with protein content 1.21 ± 0.193 mg/mL and specific activity of 0.64 ± 0.13 in 50 mL of M-9 medium with 0.3% L-asparagine as a nitrogen source, incubated at 37°C for 48 h. Similarly, glutaminase activity was estimated using L-glutamine as substrate instead of L-asparagine by direct nesslerization where no ammonia was detected and thus concluded this enzyme was free of L-glutaminase activity.(REF. justification) Further, pH and temperature of the medium were optimized for the production of L-asparaginase by this strain and it was noticed that maximum specific activity was obtained at pH 6.5 and at 37°C, while perhaps the growth was not influenced at

50°C (Supplementary Table I).

Production of L-asparaginase using industrial effluent as a substrate.

Wastes from agriculture and industry have been explored for bio energy and bio fuel production by microorganism also yielding, methane, ethanol and hydrogen peroxide as by-products.[28] Variety of cheap agro wastes and industrial wastes like corn cob, coconut oil cake, leguminous crops and palm oil effluent are being used as a substrate for the production of enzymes like L-asparaginase [29,30] and lipase.[31] Sugar cane industry influent has many applications including that may act as biocatalyst for microbial fuel cell and aconitic and lactic acid recovery. Hence, in this study an attempt was made to exploit sugar cane industry effluent as a cheaper alternate substrate to L-asparagine for the enhanced production of L-asparaginase. Based on the parameters like the presence of L-asparagine (Thin layer Chromatography) and the other parameters pH (4.85) reducing sugar content (0.43 ± 0.03 mg/mL) protein content (40.66 ± 3.8 mg/mL) and the BOD (76.6 mg/L) in the effluent was chosen as an alternate substrate for the production of L-asparaginase (Supplementary Table II). Further HPLC analysis and FT-IR analysis of the effluent was done and presence of organic nitrogen was confirmed in form of amides and amines (Supplementary Fig. B and C). In order to ensure that the microbial load of the effluent did not interfere with the enzyme activity of *Pseudomonas plecoglossicida* RS1, the effluent was sterilized by autoclaving at 15 psi for 15 minutes, thrice, on three successive days (to kill the germinating spore cells). Complete sterilization was confirmed by determining the total viable count (TVC) after each autoclaving cycle till the microbial load was brought to zero and thus it was ensured that the enzyme production was solely contributed by the isolate *Pseudomonas plecoglossicida* RS1 only. Medium containing 1% effluent was capable of producing enzyme with higher specific activity (1.9 fold) than the medium containing 1% L-asparagine, whereas no activity was detected in M-9 medium without nitrogen source as a control (Table 1).

Table 1-Screening of alternate substrates for L-asparaginase production in minimal medium by *Pseudomonas plecoglossicida* RS1.

	Media M-9	Enzyme activity (IU/mL/min)	Protein content of crude enzyme (mg/mL)	Specific activity (IU/mg)
L-Asparagine (%w/v)	0	0.00	0.008 ± 0.005	0
	0.1	0.61 ± 0.03	0.55 ± 0.06	1.02 ± 0.41
	0.3	0.87 ± 0.20	1.21 ± 0.19	0.64 ± 0.13
	1.0	1.54 ± 0.90	2.3 ± 0.30	0.62 ± 0.3
	1.5	0.76 ± 0.20	2.32 ± 0.28	0.32 ± 0.06
	2	0.58 ± 0.12	2.33 ± 0.23	0.24 ± 0.03
Effluent (%v/v)	0.1	0.31± 0.006	0.66 ± 0.06	0.47 ± 0.05
	0.5	0.88 ± 0.35	0.77 ± 0.12	1.11 ± 0.27
	1	1.43 ± 0.64	1.15 ± 0.06	1.21 ± 0.46
	1.5	0.53 ± 0.06	1.49 ± 0.14	0.37 ± 0.08
	2	0.71 ± 0.45	1.68 ± 0.31	0.49 ± 0.38

It is due the high protein content of the effluent which was substantiated with the presence of organic nitrogen by FT-IR and HPLC analysis, mimicking the natural amino acid asparagine, (Supplementary Fig. B and C) the effluent might be served as a rich nitrogen source for the enhanced production of L-asparaginase by *Pseudomonas plecoglossicida* RS1. Nitrogen supplementation had significantly influenced the production of L-asparaginase was also reported previously. [31] Sugar cane industry effluent rich in organic nitrogen content influence the production of commercially important products from microorganisms was documented [19, 20]. However, this is the first study highlighting that this sugar cane industry effluent as an alternate substrate to L-asparagine would enhance the L-asparaginase production and that may pave a way to reduce the production cost for commercialization.

Media optimization by Plackett Burman design

To enhance the potential for *Pseudomonas plecoglossicida* RS1 for L-asparaginase production, the nutrient factors such as L-asparagine, glucose, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, pH and effluent were chosen for this optimization study using statistical models PBD and CCD. Critical factors in the fermentation process and its significant interaction with each other for the practical application and validation in commercial production of this enzyme was also analyzed. Both PBD and CCD have been used previously for increasing the yield of L-asparaginse in *Bacillus licheniformis* and *Pectobacterium carotovorum* MTCC 1428. [8, 32]

PBD was used with seven factors, that were, glucose, Na_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, effluent and pH, involving 12 trials and two levels of concentration, for the response in terms of the enzyme activity (IU/mL) and specific activity (IU/mg). Variation in these factors revealed the significance of media optimization to achieve higher L-asparaginase productivity and the influence of all the factors on enzyme activity which varied from 1.17 IU/mL to 3.63 IU/mL and specific activity varied from 0.48 IU/mg to 1.68 IU/mg (Table 2).

The order of significance among the variables and the most effective factors showing high level of significance was indicated in Paretos chart. The positive and the negative influence of the factors on the L-asparaginase specific activity are also depicted further, the reference line at 2.3 (t-value limit) shows the significance level of the factors and the effect of the variables extending beyond the line was found to be highly significant by t-values (Supplementary Fig. D). L-asparaginase activity was influenced greatly by KH_2PO_4 and pH, while specific activity was greatly influenced by the factors KH_2PO_4, effluent and pH. Thus these factors were selected for further optimization by CCD whereas the other factors glucose, Na_2HPO_4, $MgSO_4.7H_2O$, NaCl and were used at their normal central values for further studies.

ANOVA analysis of the design (Table 3) also substantiated the findings of the three factors, KH_2PO_4, effluent concentration and pH as the significant with p the value < 0.05 (95% confidence) and the final equation was given with coefficient values for each of the three factors. The negative sign indicates the negative effect of the factor on the specific activity, while positive co-efficient indicates a positive influence in both enzyme and specific activity.

The Model F-value of 9.38 and 9.39 (Table 3) declares the model to be significant, and only a 0.54% chance that a "Model F-Value" so large could be due to noise. Values of "Prob > F" less

than 0.0500 indicate model terms are significant. In this case, C, F, G are significant model terms and values greater than 0.1000 indicate the model terms are not significant. As per the suggestions from the earlier reports, the confidence levels, (which is the P value expressed in percent) higher than 70% are acceptable, likewise in this experiment, variables with confidence levels were greater than 90% considered as significant. [33,34]

Table 2- Plackett Burman design matrix with predicted and observed response values of enzyme activity (IU/mL) and Specific Activity (IU/mg) of L-asparaginase produced by Pseudomonas plecoglossicida RS1

Std	A-Glucose (g/100mL)	B-Na_2HPO_4 (g/100mL)	C-KH_2PO_4 (g/100mL)	D-$MgSO_4$ (g/100mL)	E-NaCl (g/100mL)	F-Effluent (mL/100mL)	G-pH	Enzyme Activity Actual values (IU/mL)	Enzyme Activity Predicted values (IU/mL)	Specific activity Actual values (IU/mg)	Specific activity Predicted values (IU/mg)
1	0.4	0.7	0.2	0.06	0.06	1.2	6.5	3.34 ± 0.47	3.30 ± 0.53	0.96 ± 0.03	1.05 ± 0.1
2	0.2	0.7	0.4	0.04	0.06	1.2	7.1	1.43 ± 0.42	1.46 ± 0.53	0.48 ± 0.01	0.49 ± 0.1
3	0.4	0.5	0.4	0.06	0.04	1.2	7.1	1.17 ± 0.02	1.46 ± 0.53	0.54 ± 0.01	0.49 ± 0.1
4	0.2	0.7	0.2	0.06	0.06	0.8	7.1	2.21 ± 0.18	2.57 ± 0.53	0.98 ± 0.06	1.14 ± 0.1
5	0.2	0.5	0.4	0.04	0.06	1.2	6.5	1.43 ± 0.18	2.17 ± 0.53	0.58 ± 0.06	0.76 ± 0.1
6	0.2	0.5	0.2	0.06	0.04	1.2	7.1	2.29 ± 0.93	2.57 ± 0.53	1.0 ± 0.07	0.78 ± 0.1
7	0.4	0.5	0.2	0.04	0.06	0.8	7.1	3.14 ± 0.97	2.57 ± 0.53	1.0 ± 0.05	1.14 ± 0.1
8	0.4	0.7	0.2	0.04	0.04	0.8	6.5	2.99 ± 0.48	3.29 ± 0.53	1.32 ± 0.02	1.41 ± 0.1
9	0.4	0.7	0.4	0.04	0.04	0.8	7.1	1.87 ± 0.50	1.46 ± 0.53	0.90 ± 0.01	0.85 ± 0.1
10	0.2	0.7	0.4	0.06	0.04	0.8	6.5	1.90 ± 0.11	2.17 ± 0.53	0.97 ± 0.05	1.12 ± 0.1
11	0.4	0.5	0.4	0.06	0.06	0.8	6.5	3.12 ± 0.51	2.17 ± 0.53	1.36 ± 0.08	1.12 ± 0.1
12	0.2	0.5	0.2	0.04	0.04	0.8	6.5	3.63 ± 0.18	3.29 ± 0.53	1.68 ± 0.07	1.41 ± 0.1

Table 3- ANOVA for Plackett Burman Design of L-asparaginase production from *Pseudomonas plecoglossicida* RS1

	Analysis of variance (ANOVA) table (Specific Activity)						Analysis of variance (ANOVA) table (Enzyme Activity)					
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	Confidence level (%)	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	Confidence level (%)
Model	1.06	3	0.35	9.38	0.005	99.5	5.28	2	2.64	9.39	0.006	99.4
C-KH_2PO_4	0.24	1	0.24	6.85	0.031	96.9	3.74	1	3.74	13.30	0.005	99.5
F-effluent	0.37	1	0.21	10.74	0.011	98.9	-*	-*	-*	-*	-*	-*
G-pH	0.21	1	0.21	5.75	0.043	95.7	1.54	1	1.54	5.47	0.044	95.6

*factor not significant for this response

Final Equation in Terms of Actual Factors:

Enzyme Activity (IU/ml) =12.17 - 5.58 * C - 1.19* G ……(3)

Specific Activity (IU/mg) = 5.32 - 1.45 * C - 0.91* F - 0.44* G ……(4)

Where, C- KH_2PO_4, F- effluent, G - pH

Therefore, these three factors were used for the next optimization experiment using CCD, while the factors identified as insignificant were used at their normal central values.

Central Composite Design: Response Surface Methodology

Based on the results obtained from PBD, three factors, KH_2PO_4, effluent and pH were identified as critical components and CCD was performed using Design Expert 9.0 (Table 4) in order to analyse the combined effect of nutrients and to obtain a quadratic

equation in terms of these factors while other factors were added as in the un-optimized medium. Twenty runs with six replicates at the central value of the three factors KH_2PO_4, effluent and pH (0.3 g/100 mL, 1mL/100 mL, 6.8), respectively in the design, while all other runs with duplicates were performed, and the mean value of response for each run in terms of enzyme activity and specific activity obtained after 48 h incubation at 37 °C, was reported. The CCD resulted with higher specific activity (3.31 ± 0.47 IU/mL) of L-asparaginase in 1st run consisted of KH_2PO_4 (0.2 g/mL), effluent (0.8 mL) and pH (6.5) (Table.4). The data obtained from the CCD was fitted with a second order polynomial equation in order to elaborate the interaction of nutrients for L-asparaginase production by the following equation.

Enzyme activity (IU/ml) = -137.27 – (18.43 * A) – (56.52 * B) + (51.68 * C) + (9.57 * A * B) + (1 .66 * A * C) + (10.45 * B * C) – (3.89 * A^2) –10.14 B^2 – 4.67 * C^2 … (5)

and

Specific activity (IU/mg) = -51.92 - (13.31* A) - (52.35 * B) + (25.12* C) + (2.66*A * B) + (2.10 * A * C) + (6.97 * B* C) - (6.978 * A^2) + (0.62 * B^2)- (2.46 * C^2) …(6)

Where, A: KH_2PO_4, B: effluent, C: pH.

The observed values of enzyme activity and specific activity of L-asparaginase were closer to those predicted by the design model. Thus these factors were effective in achieving high production of the enzyme. Similar studies of interaction between 3 g/L of KH_2PO_4 and 0.5 g/L of $MgSO_4.7H_2O$ in the M-9 medium had shown maximum L-asparaginase activity by *Bacillus licheniformis* RAM-8. [8] Also, optimization for large scale production of L-asparaginase with carbon and nitrogen source along with little amount of mineral nutrients to achieve maximum production was also reported. [11]

The significance of the model was evaluated based on Fisher's F-test value as given by ANOVA analysis (Table 5). Here, the Model F-value of 32.63 showed the model is significant, with least chance (0.01%) that this large "Model F-Value" is due to noise. In this case B, C, BC, C^2 were significant model terms for the response specific activity and enzyme activity, C and BC were significant as their "Prob > F" was less than 0.0500. The goodness of fit of the model, given by the R squared value 0.967, (closer to 1) (for specific activity) and 0.84 (for enzyme activity) indicated the reliability of the model and hence the design is effectively conveyed in a quadratic model. Moreover, R squared value is comparable to the values previously reported. [11]

The fitted responses for the analyzed regression model were plotted as a three dimensional graph with the pair wise combination of the three factors for L-asparaginase production (Fig 1). The contour plot coupled with three dimensional surface plots explains the interaction of the parameters in terms of the selected responses. In Figure 1a., the effect of KH_2PO_4 and effluent on enzyme activity and specific activity is seen. It can be inferred that KH_2PO_4 has very little effect on both enzyme activity and specific activity when concentration is varied, however among the different concentrations of effluent used, from 1.2 (% v/v) to 0.66 (% v/v) the maximum activity was found at 0.8 (% v/v) of effluent. Similarly, from Figure 1b. and Figure 1c., it can be seen that higher enzyme activity and specific activity is observed at acidic pH (6.5) with the interaction of, KH2PO4 at 0.2g (%w/v) and effluent 0.8 (% v/v).

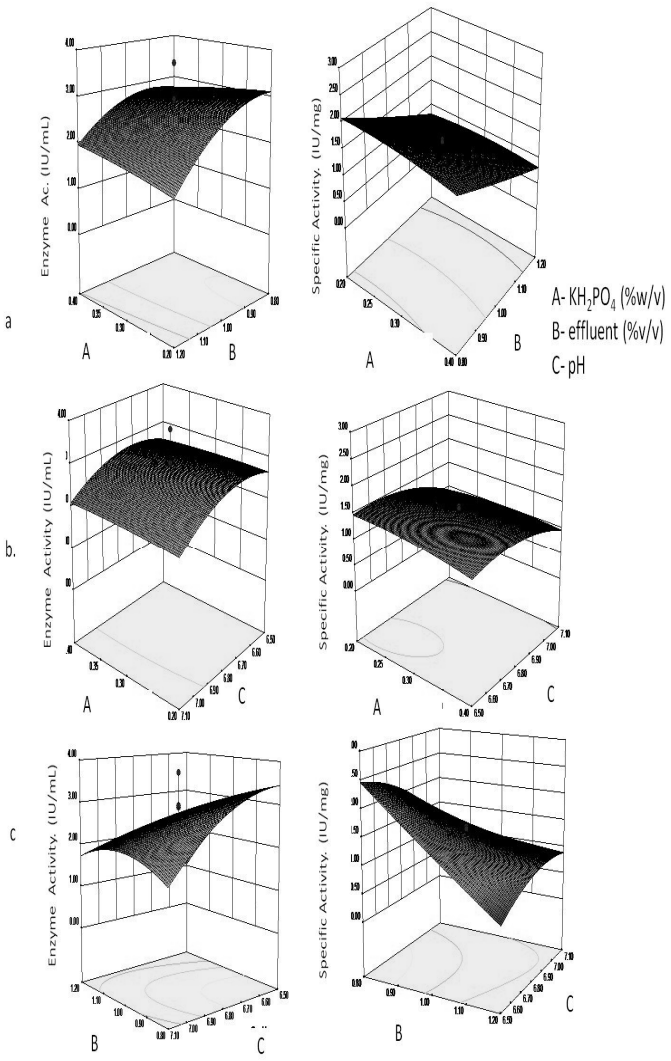

Fig. 1 Three dimensional Response Surface Curves for L-asparaginase production in terms of enzyme units per mL (IU/mL) and Specific activity (IU/mg), showing interactions between media components of M-9 medium. a. KH_2PO_4 and effluent concentration when pH was kept constant b. KH_2PO_4 and pH when effluent concentration was kept constant c. effluent concentration and pH when KH_2PO_4 concentration was constant.

Table 4 – CCD matrix with actual values and the predicted and observed response values of enzyme activity (IU/mL) and Specific Activity (IU/mg) of L-asparaginase produced by *Pseudomonas plecoglossicida* RS1

	Factor 1	Factor 2	Factor 3	Observed Response	Predicted Response	Observed Response	Predicted Response
Std	A:KH_2PO_4	B:Effluent	C:pH	Enzyme activity	Enzyme activity	Specific activity	Specific activity
	g/100mL	mL		IU/mL	IU/mL	IU/mg	IU/mg
1	0.2	0.8	6.5	3.31 ± 0.47	3.65 ± 0.51	2.54 ± 0.09	2.56 ± 0.19
2	0.4	0.8	6.5	2.84 ± 0.22	3.17 ± 0.51	1.98 ± 0.15	2.23 ± 0.19
3	0.2	1.2	6.5	0.62 ± 0.05	0.85 ± 0.51	0.34 ± 0.03	0.45 ± 0.19
4	0.4	1.2	6.5	0.83 ± 0.26	1.14 ± 0.51	0.36 ± 0.09	0.33 ± 0.19
5	0.2	0.8	7.1	1.69 ± 0.35	1.73 ± 0.51	1.06 ± 0.11	1.19 ± 0.19
6	0.4	0.8	7.1	1.34 ± 0.17	1.46 ± 0.51	1.12 ± 0.11	1.11 ± 0.19
7	0.2	1.2	7.1	1.43 ± 0.06	1.44 ± 0.51	0.90 ± 0.13	0.75 ± 0.19
8	0.4	1.2	7.1	1.92 ± 0.33	1.93 ± 0.51	0.81 ± 0.14	0.89 ± 0.19
9	0.13	1	6.8	2.87 ± 0.37	2.67 ± 0.51	1.36 ± 0.09	1.34 ± 0.19
10	0.47	1	6.8	2.98 ± 0.32	2.69 ± 0.51	1.30 ± 0.04	1.17 ± 0.19
11	0.3	0.66	6.8	2.94 ± 0.35	2.62 ± 0.51	2.68 ± 0.06	2.5 ± 0.19
12	0.3	1.33	6.8	0.84 ± 0.49	0.67 ± 0.51	0.51 ± 0.21	0.55 ± 0.19
13	0.3	1	6.2	2.63 ± 1.20	2.08 ± 0.51	1.34 ± 0.12	1.18 ± 0.19
14	0.3	1	7.3	1.06 ± 0.12	1.13 ± 0.51	0.46 ± 0.07	0.48 ± 0.19
15	0.3	1	6.8	2.91 ± 2.91	2.79 ± 0.51	1.52 ± 0.08	1.45 ± 0.19
16	0.3	1	6.8	2.95 ± 0.35	2.79 ± 0.51	1.24 ± 0.04	1.45 ± 0.19
17	0.3	1	6.8	3.72 ± 0.21	2.79 ± 0.51	1.54 ± 0.15	1.45 ± 0.19
18	0.3	1	6.8	2.51 ± 0.28	2.79 ± 0.51	1.48 ± 0.12	1.45 ± 0.19
19	0.3	1	6.8	2.45 ± 0.07	2.79 ± 0.51	1.44 ± 0.15	1.45 ± 0.19
20	0.3	1	6.8	2.10 ± 0.31	2.79 ± 0.51	1.50 ± 0.06	1.45 ± 0.19

Table 5- ANOVA for Response Surface Quadratic Model for L-asparaginase production from *Pseudomonas plecoglossicida* RS1

	Analysis of variance (ANOVA) table (Specific activity)						Analysis of variance (ANOVA) table (Enzyme activity)					
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F		Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	7.46	9	0.83	32.63	< 0.0001	Significant	13.62	9	1.51	5.92	0.005	Significant
A-KH_2PO_4	0.03	1	0.03	1.34	0.2741		0.001	1	0.001	0.002	0.96	
B-effluent	4.62	1	4.62	181.70	< 0.0001		4.59	1	4.58	17.95	0.001	
C-pH	0.58	1	0.58	22.82	0.0007		1.08	1	1.08	4.24	0.06	
AB	0.023	1	0.02	0.89	0.3670		0.29	1	0.29	1.14	0.31	
AC	0.03	1	0.03	1.26	0.2879		0.02	1	0.019	0.07	0.78	
BC	1.40	1	1.40	55.08	< 0.0001		3.14	1	3.14	12.30	0.005	
A^2	0.07	1	0.07	2.76	0.1275		0.02	1	0.021	0.08	0.77	
B^2	-	1	-	0.35	0.5654		2.37	1	2.3716	9.28	0.01	
C^2	0.17	1	0.70	27.73	0.0004		2.55	1	2.55	9.98	0.01	
Lack of Fit	0.05	5	0.01	3.33	0.11	not significant	0.98	5	0.19	0.62	0.69	not significant

Verification of the model

The verification of the model was performed to validate the above model for the reproducibility of the results. The significant increase in the enzyme activity (as the response) obtained in media components of standard order 1 of CCD design consisted of KH_2PO_4 (0.2 g % w/v), effluent (0.8 mL% v/v), pH (6.5). Hence the run with above components at optimum value would be considered as the optimized media and all other runs of the design that had not shown significant increase in the response were neglected for further verification. The enzyme activity (3.25 ± 0.11 IU/mL) and specific activity (2.77 ± 0.06 IU/mg) obtained after optimization was consistent and very close to the predicted model (3.65± 0.51 IU/mL and 2.56 ± 0.19 IU/mg) with a 4.5 fold increase in enzyme activity and 4.3 fold increase in specific activity compared to the M-9 medium provided with 0.3% L-asparagine (enzyme activity 0.73 ± 0.08 IU/mL and specific activity 0.64 ± 0.03 IU/mg).

Purification and characterization of enzyme

The purification process of L-asparaginase some time may lead to loss in its activity and making it worthless in commercial applications. Hence, L-asparaginase activity was continuously analysed in all the purification steps and all purification steps were carried out at 4˚C and the column chromatographic runs were analyzed for protein at 280 nm, while the enzyme activity was estimated by direct nesslerization. Ammonium sulphate precipitation (80%) had retained the enzyme activity and after the dialysis against Tris-HCl buffer at pH 8.6 the activity enzyme was also found to be stable. The enzyme was further purified to homogeneity by DEAE Sepharose chromatography, which was verified by both native PAGE and SDS PAGE analysis (Fig. 2). The fold purification of L- asparaginase was 4.36 with 19 % recovery and summary of fold purification achieved is given in Table 6.

Table 6- Summary of the various steps involved in purification of L-asparaginase produced by *Pseudomonas plecoglossicida* RS1

Enzyme fraction	Vol mL	Total Units IU	Total protein mg	Specific Activity IU/mg	Fold purification	% yield
Crude enzyme	100	211.30	97.90	2.16 ± 0.03	-	100
Ammonium sulphate ppt. (80%)	18	55.01	16.00	3.44 ± 0.30	1.59	26
DEAE Sepharose	6	41.13	4.32	9.43 ± 0.40	4.36	19

The purity and approximate size of L-asparaginase from DEAE Sepharose was assessed by Native PAGE revealed that the single distinct band of protein having ~230 kDa was compared with multiple bands in the crude fraction. Also SDS PAGE analysis exhibited the size of the subunit of the protein as a single band with the molecular weight of ~46 kDa, which showed evidence of that the L-asparaginase is a homo-pentamer. The L- asparaginase of gram negative bacteria *Serratia* sp. exists as pentamer or hexamer with a average molecular weight of 170 to 180 kDa as reported by Stern et al., (1976).[35] where as the L-asparaginases was found to be a homotetramer with identical sub units (38000 Da) in *E. coli* [36]. The molecular size and subunits of L-asparaginase in *Pseudomonas* vary from a single subunit of 34 kDa to 33 kDa under non-denaturing and denaturing conditions respectively. [14] L-asparaginase also exists as a monomer of 160 kDa in size in *Pseudomonas* as reported by El-Bessoumy et al., (2004)[9]. Thus, L-asparaginase shows a wide structural variation in the subunits of the above mentioned bacteria.

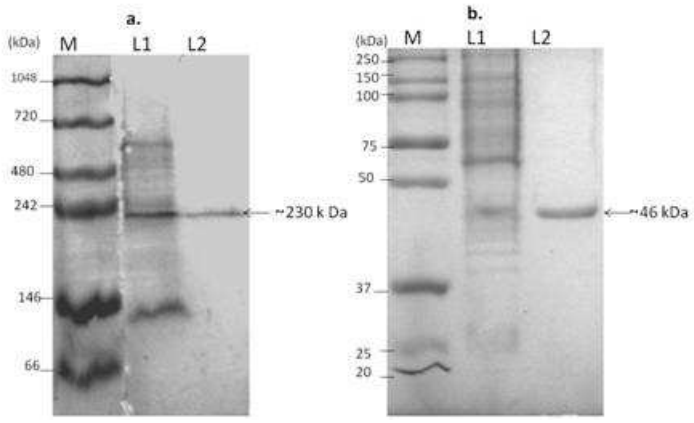

Fig.2 Electrophoretic analysis of L-asparaginase produced by *Pseudomonas plecoglossicida* RS1 (a) Native PAGE on 7.5 % resolving gel (b) SDS PAGE on 12% resolving gel M- Marker, L1- Crude enzyme, L2- Purified enzyme

Effect of pH, temperature and metal ions on activity and stability of purified L-asparaginase

Characterization of purified enzyme was performed using different effectors like pH, temperature, and metal ions. The maximum enzyme activity (6.45 ± 0.87 IU/mL) was observed at pH 8.5 whereas the enzyme activity was decreased in acidic conditions. The stability of the enzyme (residual activity) when stored at 4°C for 24 h with buffers of different pH (4-9.5), was maximum (100%) at 8.5 and decreased significantly in the acidic range, whereas it retained upto 80% activity at pH 9.5 (Fig. 3a). It was also already reported that the L-asparaginase activity was stable at pH 7.5 to 9 in *Pectobacterium carotovorum* MTCC1428 [26], *Pseudomonas aeroginosa* 50071 [9] and *Pseudomonas aeruginosa* 10145 [34]. Likewise the optimum temperature for the enzyme activity (10.74 ± 0.08 IU/mL) was found at temperature 55°C for *Pseudomonas plecoglossicida* RS1 while the temperature beyond 15°C to 55°C the enzyme activity was decreased to 50 % (Fig. 3b). This is a noteworthy feature to compare with the previous reports of stability of L-asparaginases at 45°C to 60°C, 75% of enzyme activity was lost. [26] The impact of various effectors on the enzyme was also demonstrated using different effectors like (Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}, EDTA, L-Cystine, L- Histidine, Mercaptoethanol and SDS. Of the various effectors $Na^+$and mercaptoethanol were slightly inducing the enzyme activity where as Hg^{2+} $Ca^{2+}$and Zn^{2+} had shown highly detrimental activity than the control and other effectors (Fig. 3c) and this effect could be due to the presence of sulfhydryl group(s) in enzyme substrate complex. These results were corroborated with the *Erwinia carotovora* L-asparaginase activity in the presence of thiol compounds reported by Warangkar and Khobragade (2010)[25]. Furthermore, EDTA did not result with large inhibition, indicating that the enzyme may not be a metalloprotein as suggested by Kumar et al., (2011)[26].

Kinetic parameters of L-asparaginase

In order to find the affinity between the enzyme substrate system, determination of K_m and V_{max} values are important. Obviously, L-asparaginase from different microorganisms may also differ in their substrate affinities and that would execute different physiological functions in enzyme activity. The K_m of the purified L-asparaginase produced by *Pseudomonas plecoglossicida* RS1 was 2.25 ± 0.61 mM and its V_{max} was 8.95 ± 0.8 IU/mL/min (Fig. 4). K_m values for L-asparaginase produced by *C. glutamicum* and *E. coli* (2.5 mM and 3.5 mM) was reported by Willis and Woolfolk, (1974) [37] while the lower values of K_m (0.074 mM) by *Vibrio succinogenes* and 0.657 mM by *Pectobacterium carotovorum* MTCC 1428 was also reported previously by Kumar et al., (2011)[26].

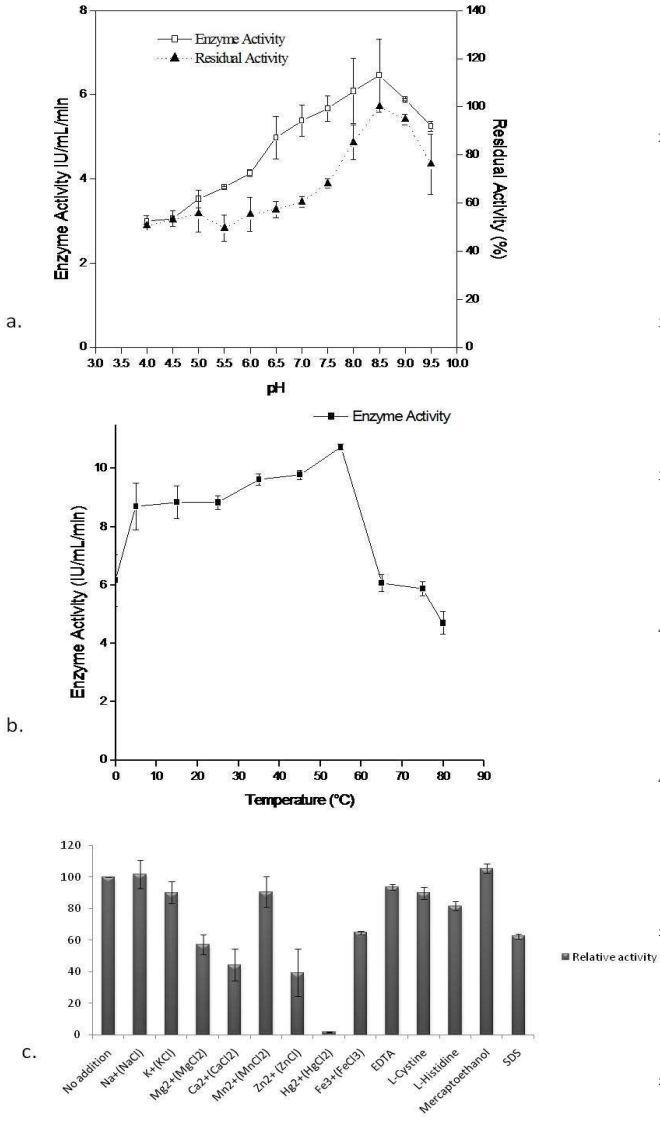

Fig 3. a. Effect of pH of assay buffer at 37°C on enzyme activity and stability of purified L-asparaginase when stored at 4°C for 24 h at different pH 3.5 to 9.5 b. Effect of temperature on L-asparaginase activity at different temperature from 0 to 80ºC after 30 min incubation. c. Influence of various effectors on the activity of purified L-asparaginase.

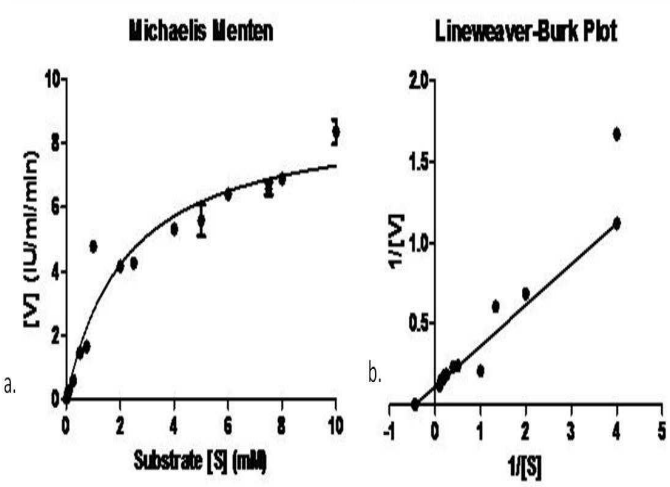

Fig.4. (a). Michaelis Menten Plot of reaction velocities (V) given by enzyme activity per minute per mL of the enzyme vs. substrate conc. (S) given by mM. Parameters V_{max} 8.95 IU/mL/min and K_m 2.25 mM were calculated by non-linear regression analysis. (b) Corresponding Lineweaver burk plot.

CONCLUSION

This study demonstrated glutaminase free L-asparaginase production by *Pseudomonas plecoglossicida* RS1 in M-9 medium provided with sugar cane industry effluent as an alternate substrate to L -asparagine. The media optimization studies using PBD and CCD also enhanced the production and the enzyme was further purified to homogeneity with the molecular size of ~230kDa on native PAGE and subunit size of ~46kDa on SDS PAGE. The enzyme characterization studies exhibited effective activity and stability to wide range of pH, temperature and effectors. Conclusively, glutaminase free L-asparaginase can be produced using effluent as an alternate substrate for the cost effectiveness and its therapeutic applications. The therapeutic application of the enzyme produced would further be evaluated by testing on T-cell cancer cell lines and animal model studies.

Acknowledgements

Authors thank the DST-SERB- SB/EMEQ-128/2013 for the financial support to PV; CSIR-UGC (No.17-06/2012(i) EU-V) fellowship to GS and DST-PURSE program for instrumentation facility.

Author Contributions

PV and BA design the study and analysed the data and wrote manuscript. GS and SMB performed the experiments, analysed the data and wrote the manuscript. PP was involved in the purification and characterization experiments. All the authors have contributed for writing the manuscript and have approved the final manuscript.

Notes and references

[a] *Department of Molecular Microbiology, Madurai Kamaraj Univeristy Madurai, India. Fax: 914522459105; Tel: 91452-2459115;*

[b] *Department of Genetic Engineering ,Madurai Kamaraj Univeristy Madurai, India. Fax: 914522459105; Tel: 91452-2459115.*
[a*] *Corresponding Author- Department of Molecular Microbiology, Madurai Kamaraj Univeristy Madurai, India. Fax: 914522459105; Tel: 91452-2459115;*

† Electronic Supplementary Information (ESI) available: [Phylogenetic tree, Characterization of effluent, Paretos chart].
See DOI: 10.1039/b000000x/

1 J. D. Broome, *J. Exp. Med.*, 1968, **127**,1055.
2 E. A. Boyse and L. J.Old, H. A.Campbell, L. T. Mashburn, *J Exp. Med.*, 1966, **125**, 17.
3 L . T Mashburn and J. C . Wriston., *Arch. Biochem. Biophys*., 1964 **105**: 450.
4 Y. Ghasemi, A. Ebrahiminezhad, S. R. Amini, G. Zarrini, M. B. Ghoshoon, M. J. Raee, M. H. Morowvat, F. Kafilzadeh and A. Kazemi, *Am. J. Biochem. Biotechnol.,* 2008, **4** ,422.
5 T. Tosa, R. Sano, K .Yamamoto and M. Nakamura*, Am. Soc. Microbiol.*, 1971, 387.
6 C. K. Venil., K. Nanthakumar, K. Karthikeyan and P. Lakshmanaperumalsamy, *Iran. J. Biotechnol.* , 2009, **7**, 1.
7 S. Ghosh, S. Murthy, S. Govindasamy and M. Chandrasekaran Sustainable Chemical Processes, 2013, **1**,9.
8 R. V. Mahajan, S. Saran , K. Kameswaran a, V. Kumar and R. K. Saxena, *Bioresour. Technol.*, 2012, **125,** 11.
9 A. A . El-Bessoumy, M. Sarhan and J .Mansour , *J. Biochem. Mol. Biol*., 2004,**37**,387.
10 S. Amena, N. Vishalakshi , M. Prabhakar, A. Dayanand and K. Lingappa, *Braz. J. Microbiol.,* 2010, **41**, 173
11 R. Usha, K. K. Mala., C.K Venil and M. Palaniswamy , *Pol. J. Microbiol*, 2011,**60**, 213.
12 B. Gurunathan and R. Sahadevan, *J. of Microbiol. Biotechnol.*, 2012, **22**, 923.
13 J. B. Howard and F. H. Carpenter, *The J. Biol. Chem.*, 1972, **247**, 1020.
14 S. Manna, A. Sinha, R. Sadhukhan, and S. L. Chakrabarty, *Curr. Microbiol.* , 1995,**3**,291-8
15 G. Ollenschläger, E. Roth, W. Linkesch, S. Jansen, A. Simmel and B. Mödder, *Eur. J. Clin. Invest.* , 1988,**18**,512.
16 L. N. Ramya , M. Doble, V. P. B. Rekha and K. K. Pulicherla, *Appl. Biochem. Biotechnol.*, 2012,**167**,2144.
17 U. K .Narta, S. S. Kanwar and W. Azmi, *Crit. Rev. Oncol. Hematol.,* 2007,**61**,208.
18 S. Kumar, K. Pakshirajan., V. Venkata Dasu, *Process Biochem.* ,2010, **45**, 223.
19 S. R . Rao, R. S. Prakasham, K. Krishna Prasad, S. Rajesham, P. N. Sharma, and V. Rao *Process Biochem.*, 2004, **39**, 951.
20 R. Karpagam, , K. J. Raj, , B. Ashokkumar, , P. Varalakshmi, *Bioresour. Technol.*, 2015, http://dx.doi.org/10.1016/j.biortech.2015.01.
21 R. S. Prakasham, S Rao, S. R. Rao and P. N. Sarma, *Biotechnol. Prog .,* 2005, **21**, 1380.
22 R . Gulati, R. K. Saxena and R. Gupta, *Lett. Appl. Microbiol.,* 1997, **23**,6.
23 J. B Sumner, *J. Biol. Chem*. 1921, 47, 5.
24 R. L. Plackett and J.P. Burman , *Biometrika,* 1946, **33**, 305.
25 U. K. Laemmli, *Nature* 1970. **227**, 680.
26 S. C. Warangkar and C. N. Khobragade, *Enzyme Res.,* 2010, ID-**165878**, 1–10.
27 S. Kumar , V. Venkata Dasu and K. Pakshirajan, *Appl. Biochem. Biotechnol.*, 2011,**163**,327.
28 D. Pant, A. Singh, G. Van Bogaert,S. I. Olsen,P. S. Nigam, L. Dielsa and Karolien Vanbroekhovena, *RSC Adv.*, 2012, **2**, 1248.
29 E. A. Makky,Y. C. Loh and Md. R. Karim, *Biocatal. Agric. Biotechnol.*, (2014), **3**, 265.
30 A. Mishra, *Appl. Biochem. Biotechnol.*, 2006. **135,** 33.
31 A. Salihu, M. Z. Alam, M. I. AbdulKarim, H. M. Salleh, *Afr. J. Biotechnol.*, 2011, **10**, 18704.
32 S. Kumar, K. Pakshirajan and V. Venkata Dasu , *Appl. Microbiol. Biotechnol.* ,2009, **4**,477.
33 R.A. Stowe and R.P. Mayer, *Ind. Eng. Chem.*, 1966, **58** ,36 .
34 Y. R. Abdel-Fattah and Z. A. Olama, *Process Biochem.*, 2002,**38,** 115._
35 M. L. Stern, A.W. Phillips, A. J. Gottlieb, *J Bacteriol.* 1976, **125**, 719.
36 H. J Muller and J. Boos .*Critical Rev. Oncol.:Hematol*., 1998, **28** ,97.
37 R. C.Willis, C.A. Woolfolk, *J.Bacteriol.,*1974. **118**, 231.