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Optimization of Solid State Fermentation Conditions for Biosynthesis of L-Asparaginase Enzyme using *Wautersiaeutropha* NRRL B-2804

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Abstract: ***This article reports on developing media by optimizing parameters for biosynthesis and isolation of bio-molecule 'L-Asparaginase' from 'WautersiaeutrophaNRRL B-2804' by solid state fermentation technology. Different agricultural solid waste substrates including Vigna radiate bran (green gram bran), Cajanuscajanbran (red gram bran), Cicerarietinumbran (black gram bran), and Glycine max bran (soyabean bran) have been screened in the study. Among the substrates studied Vigna radiate bran gave maximum production of L-Asparaginase of 0.67 U/gds. Parameters including inoculums media, seed age, percentage and concentration of impregnating media, fermentation period, cell disruption pH, cell disruption time, compositions of impregnating media, inoculums size, carbon source and nitrogen source were studied and optimized. The study showed maximum enzyme activity of 1.38 U/gds after one factor at a time optimization method. The parameters including Glycerol (0.5 % to 1.5 % w/w), L-Asparagine (0.5 % to 1.5 % w/w) and Tryptone (0.25 % to 0.75 % w/w) were optimized using Response Surface Methodology study (RSM) based on central composite design (CCD). By using the surface plots and response optimizer of Design Expert Version 6.0.8 (Stat-Ease, Minneapolis, MN 55413) software the maximum enzyme activity of 1.42 U/gds was obtained when glycerol, L-Asparagine and Tryptone concentration was 1.00 % w/w, 1.00 % w/w and 0.25 % w/w respectively. The ammonium sulphate precipitation gave purification factor (P.F.) of 3.39 and on subjecting the obtained precipitate to dialysis followed by microfiltration and Ultra filtration the P.F. was increased to 5.76 times.***

Keywords: *Wautersiaeutropha 1*, Solid state fermentation 2, RSM (Response Surface Methodology) 3, L-Asparaginase 4, L-Asparagine 5, One Factor At a Time (OFAT) 6

1. Introduction

Enzymes are bio-molecules or proteins that catalyze substrate specific chemical reactions to produce products. Among them, L-asparaginase (amido hydrolase, E.C. no. 3.5.1.1) is an enzyme which converts L-Asparagine to L-aspartic acid and ammonia. It is one of the enzymes that have a wide range of applications in pharmaceutical, food and agricultural, chemical, fertilizer industry and also emerged as potent health care agent (1).

The therapeutic potential of this enzyme is well established, as it has remarkable biopharmaceutical application in the treatment of Acute Lymphoblastic Leukemia (ALL) and in many other clinical experiments relating to tumor therapy and chemotherapy. The statistical data by American Society on Cancer Research (ASCR) in the past decades has raised the importance of studying L-Asparaginase as an important biopharmaceutical for those specific cases where blood cells become cancerous, such as in ALL. The enzyme, L-Asparaginase cuts off the supply of asparagines in the blood and the cancer cells die as they are unable to synthesize their proteins. Researches have shown that tumor cells take L-asparagine from blood circulation or body fluid sine it cannot synthesize L-asparagines. The presence of L-asparaginase enzyme as chemotherapeutic agents may degrade the L-asparagine present in blood which in turn leads to starvation of tumor cells and cell death. This property of L-Asparaginase can be exploited as a sensitive tool for the treatment of cancer (1,2).

L-Asparaginase was produced throughout the world by a common practice of submerged fermentation (SF). But, the major shortcomings of SF are low product concentration, cost intensiveness, handling and disposal of large volumes of spent aqueous discharge during processing (3). With respect to this, solid state fermentation (SSF) has emerged as an effective technique to increase the product yield at low capital cost and it also offers many other advantages (4). Usually substrates used for SSF are water insoluble lingo-cellulosic agricultural materials wastes to which microbes attach and degrade the substrate with their enzymatic actions (5). Thus, use of solid agricultural waste makes the SSF environmental friendly.

The enzyme L-Asparaginase is widely distributed in nature from bacteria to mammals. L-Asparaginase was first isolated from guinea pig serum which proved to be inhibitory to certain animal tumors (6). But, the low levels of L-Asparaginase present in guinea pig serum made it necessary to seek a more practical source of this anti-neoplastic enzyme. Microbial enzymes are preferred over animal or plant sources due to their economic production, consistency, stability of enzymes and many other advantages (7). The production of L-Asparaginase has also been studied in *Serratiamarcescens*, *Erwiniacarotovora, Escherichia coli*, *Enterobacteraerogenes, Pseudomonas aeruginosa,* and *Bacillus subtilis*. Although, bacterial sources were helpful in producing high yield of the enzyme, the clinical use of the certain microbial L-Asparaginase was associated with pronounced toxicity, allergic reactions and anaphylaxis (8, 9). However, toxic side effects of the currently used clinical preparations have necessitated the search for alternative microbial sources.

A wide range of microorganisms such as fungi, yeasts, and bacteria have proved to be beneficial sources of L-Asparaginase.Production of L-Asparaginase from fungal culture including *Aspergillusniger* using agricultural waste in SSF has been reported by Mishra (10). Whereas, Bessoumy et al., (2004) reported the production of L-Asparaginase from *Pseudomonas aeruginosa* 50071 using SSF (10). In most of the microorganisms, L-Asparaginase accumulates as an intracellular (periplasmic, cytoplasmic and membrane

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bound) product. *Wautersiaeutropha* produces L-Asparaginaseintracellularly, alkali soluble enzyme with iso-electric pH of 8.6. L-Asparaginase from *W.eutropha (Alkalegenouseutrophus, Hydrogenomonaseutrophus*-NRRL B-2804) showed striking differences with respect to allergenic reactions (11) compared L-Asparaginase production with the microbial sources including *Enterobacteraerogenes* (12) *Scharomycescerevisiae* (13) and *Acinetobacterglutaminasificans* (14). *W.eutropha* was thus selected for the production of L-Asparaginase enzyme under SSF.

For the commercial production of enzyme, selection of superior strain, treatment of the substrate by means of mechanical or chemical methods, optimization of the conditions, substrate impregnation with various external nutrients with suitable moisture content enhances the growth of microbes and enzyme harvesting protocol is a crucial step. Statistical methodologies involved used mathematical models for designing fermentation processes and analyzing the process results (15). RSM is a powerful mathematical model with a collection ofstatistical techniques wherein, interactions between multiple process variables can be identified with fewer experimental trials. It is widely used to examine and optimize the operational variables for experiment designing, model developing and factors and conditions optimization (16). Hence, RSM study was conducted to optimize the conditions for maximum L-asparaginase production in this work.

By considering all those aforementioned aspects, the main aim of the present study was to determine the optimal level of the process variables by both one factor at a time (OFAT) and Response surface methodology (RSM) methods for the production of L-asparaginase enzyme for therapeutic use with novel*W. eutropus*(NRRL B-2804) by selecting low cost substrate and through SSF method. Initially, substrates screening for *W. eutropha* growth and maximum L-asparaginase enzyme production was carried out with four different locally available pretreated solid agricultural waste materials. Further, optimization of media conditions for SSF was studied with one factor at a time (OFAT) method. Later, a statistical tool, central composite design (CCD) of RSM for optimization of solid state fermentation parameters for L-asparaginase was conducted. Finally, partial purification of L-Asparaginase enzyme using ammonium sulphate precipitation, dialysis, microfiltration and ultra-filtration were carried out to improve the purity of the isolated L-Asparaginase enzyme.

2. Materials and Methods

Chemicals and substrates:

Nutrient agar, tryptone, yeast extract, glucose, potassium dihydrogen phosphate (KH_2PO_4), glycerol, disodium hydrogen phosphate (Na_2HPO_4), peptone, sodium citrate, diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), magnesium sulphate$MgSO_4.7H_2O$, ferrous sulphate $FeSO_4.7H_2O$, dipotassium hydrogen phosphate (K_2HPO_4), nutrient broth, trichloroacetic acid (TCA), Tris base, sodium chloride NaCl, sodium hydroxide NaOH, ethanol, methanol, acetic acid, hydrochloric acid, Nesslers reagent, L-Leucine, L-Arginine, L-Aspartic acid, Glycine, L-Alanine, L-Glutamine, L-Asparagine, sodium lauryl sulphate (SDS), acryl amide, bisacrylamide were laboratory and/or reagent grade which were purchased from HiMedia Laboratories Pvt. Ltd. Four agricultural solid substrates including *Vigna radiate* bran, *Cajanuscajan*bran, *Glycine max* bran, *Cicerarietinum* bran were procured from the local market. Dialysis membrane was purchased from Fisher Scientific. Ultra-filtration assembly Kvick start Cassettes was procured from GE Healthcare. The experiments were performed in year, 2010-2011, at Pharmacology department and DBT- ICT center of Institute of Chemical Technology, Matunga.

Substrate selection study

The enzyme production was studied with four types of pre-treated agricultural solid substrates including, *Vigna radiate* bran, *Cajanuscajan*bran, *Glycine max* bran, *Cicerarietinum* bran. A blank substrate was studied without the microbial strain sample and the value of enzyme activity was subtracted from the enzyme activity achieved through the sample. The process was followed elsewhere unless otherwise stated.

Carbon, hydrogen, nitrogen (CHN) and protein

The nutrient analyses in terms of CHN were analyzed by Dumas method (17) and protein concentration was determined by Bradford assay (18). The values of CHN and protein content of substrate are presented in Table 1A.

Microorganism and culture conditions

The microbial strain of *Wautersiaeutropha*NRRL-B-2804 was procured from Northern Regional Research Laboratory NRRL, USA and was maintained as glycerol stocks at -80 °C which was further cultured on nutrient agar (pH 7.4). The slants were incubated at 30 ± 2 °C for 24 hours and were sub-cultured weekly. Single colony from the slant was selected for the seed culture media and seed age development study. This seed culture was used in the further inoculation in SSF.

Fermentation procedure and extraction of L-Asparaginase enzyme

To prepare fermentation media,agriculture solid waste (substrate) was pretreated. The pretreatment involved soaking of the substrate in 1.0 M NaOH solution (1 g substrate with 1 ml NaOH) for 24 hrs and later was thoroughly washed with distilled water 3-4 times till neutral pH was attained. After separating the water through mesh, the substrate was dried in oven at 60 ± 5 °C for approximately 4-5 hours. Further, particle size of the dried substrate was reduced by grinding in the household mixer. For preparation of fermentation flask, 5 g of the pretreated substrates (*Vigna radiate* bran, *Cajanuscajan*bran, *Glycine max* bran, *Cicerarietinum* bran) was individually added to 250 ml conical flask and was autoclaved at 121°C for 20 minute at 15 psi. Subsequently after cooling, the flask were kept in UV-light for 30 minute and then the moisture content was adjusted to 25% w/w using distilled water which was further inoculated with the overnight seed culture (OD 0.6-0.8 at 600 nm) in aseptic zone. This was thoroughly mixed with a sterile glass rod and was incubated for fermentation at room temperature (30 ± 2 °C) with intermediate shaking of the flask.The cells and substrate was separated using 50 ml tris-buffer (pH 7) containing 0.05% of Tween 20 and by sieving the mixture of solid substrate and cell suspension using the sieve. The cell suspension was then centrifuged at 4°C at 4000 rpm to obtain the cell pellet. The enzyme was isolated from the cell pellet using alkaline buffer solution of sodium borate of 5 ml at pH 8.5 and temperature of 4 ± 2°C at 180 RPM on orbital shaker incubator for 30

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minutes to disrupt the cell (19) Then, the suspension was centrifuged and the aqueous supernatant solution was immediately assayed with Nesselerization method to determine the L-Asparaginaseactivity (20). This process was followed in the each experiment unless otherwise stated.

Analysis procedure of L-Asparaginase activity: L-Asparaginase activity was measured by Nesslerization method (20). The reaction mixture consisted of 0.2 ml of 0.05 M Tris-HCl (pH 8.6), 1.7 ml of 0.01 M L-Asparagine and 20µl of appropriately diluted enzyme. After incubation for 10 minutes at 37 ^{o}C, the reaction was stopped by the addition of 0.1 ml of 1.5 M TCA (Trichloroacetic Acid). The contents were clarified by centrifugation and to 2.5 ml of the clear supernatant an equal volume of de-ionised water was added. To this mixture, 0.5 ml of Nessler's Reagent was added and it was incubated at 37 ^{o}C for 10 minutes. The absorbance was taken at 436 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol of ammonia at 37 ^{o}C.

Procedure for the Optimization of solid-state fermentation conditions for enzyme production: To elucidate the relative efficiency of the selected agro solid substrate, a series of fermentation experiments were conducted in sequential order. The strategy adopted for optimization of various process parameters influencing L-Asparaginase activity included consecutive evaluation of parameters. Initially, one parameter was studied and it was further incorporated at its optimum level in the subsequent optimization experiments. Details of optimization experiments for various **process parameters** are discussed below. Each of the experiment was performed in triplicate.

Selection of seed culture media and seed age: Figure 1 A and 1B Seed culture mediawere prepared in four groups: (a) TGY: tryptone 5 g/L, glucose 1 g/L, K_2HPO_4 1g/L, yeast extract 5 g/L,; (b) GYP: glycerol 0.1 g/L, yeast extract 0.04 g/L, peptone 0.05 g/L, pH 7; (c) Nutrient broth and (d) semi-synthetic media includes sodium citrate 0.075 g/L, $(NH4)_2HPO_4$ 0.02 g/L, $MgSO_4.7H_2O$ 0.02 g/L, $FeSO_4.7H_2O$ 0.0002 g/L, K_2HPO_4 0.00125 g/L and yeast extract 0.015 g/L. Chemicals from each group were added individually to four different Erlenmeyer flask in 250 ml distilled water (pH -7 adjusted with 1N NaOH and/or HCl) and sterilized in an autoclaved at 121°C for 20 min at 15 psi. Seed age studies were carried out individually in six different Erlenmeyer flask containing 100 ml seed culture medium. Each flask was kept for 8 hrs, 12 hrs, 24 hrs, 36 hrs, 48 hrs, 56 hrs respectively and was shaken at 180 rpm on orbital shaker at room temperature (30 $\pm$ 2 ^{o}C). Optical density of the culture was measured at 600 nm at specified time intervals, then the culture was centrifuged at 4000 rpm for 15 min at 4 ºC, and pellet was subjected to extraction process mentioned earlier.

Effect of initial moisture content: Samples containing seven different moisture levels (5%, 10%, 15%, 20%, 25%, 30%, and 40% v/w) were prepared by adjusting moisture content on 5 gm of studied substrate with optimized impregnating media. Moisture content (MO) was calculated theoretically and practically as:

$$\% \text{ Theoretical moisture content (MO)} = [M/(M+S)] \times 100$$

$$\% \text{ Practical MO} = \frac{(\text{Weight of plate with the sample - weight of plate after drying})}{(\text{Weight of plate with the sample - weight of empty plate})} \times 100$$

Where, MO=Moisture content, M=% moisture content, S=weight in grams of solid dry substrate.
The enzyme activity measured was divided by the weight of the dry substrate and was denoted as U/gds.
Cell mass obtained after fermentation was calculated as:

$$\text{g of culture/ g of substrate} = \frac{(\text{Weight of total cell pellet obtained after SSF})}{(\text{Weight of substrate taken for fermentation})}$$

Effect of fermentation period: To determine the optimum fermentation period, experiments were carried out with 5 sets of flasks, each for a period of 24 hrs, 36 hrs, 48 hrs, 72 hrs, and 96 hrs after which the extracted samples were analyzed for L-Asparaginase production.

Effect ofpH and time for cell disruption: The enzyme was isolated using alkaline buffer solution of sodium borate buffer of pH 8, 9, 10, 11, 12 at 5 ± 1°C on a shaker incubator to disrupt the cell. Following pH optimization, time as a factor to obtain high enzyme activity was studied with 4 set of flasks for 15 min, 30 min, 45 min and 60 min respectively.

Effect of impregnating media/ nutrient supplement: Composition of impregnating medium for adjusting the moisture content included basal salt solution (BSS). The composition of the diluents were as follows: **BSS-I**: Na_2HPO_4 0.47 %, KH_2PO_4 0.45 %, $MgSO_4$ 0.05 %; **BSS-II**: $(NH_4)_2NO_3$ 2.3 %, $MgSO_4$ 7.H_2O 0.46 %; **BSS-III**: Na_2HPO_4 0.47 %, KH_2PO_4 0.454 %, $MgSO_4$ 0.05 %, $(NH_4)_2NO_3$ 2.3 %, sodium chloride 0.46 %, Malt extract 0.5 % and **Control**: Distilled water 100 %

Effect of inoculums volume: The enzyme production was studied by varying the inoculums volume as 1ml, 2ml, 3 ml, 4 ml, 5 ml, 6 ml and 7 ml of bacterial culture.

Effect of supplementary carbon sources: Various carbon sources like Glucose, Sorbitol, Glycerol, Fructose, Sucrose and Lactose were incorporated at a concentration of 1% (w/v) in the BSS-II and their effect on L-Asparaginase activity and *W. eutropha* growth was analyzed.

Effect of supplementary nitrogen sources (complex nitrogen sources): To study the effect of various nitrogen sources on L-Asparaginase production, four different nitrogen sources namely tryptone, malt, yeast extract and peptone were selected and incorporated in 1 % w/v of the BSS-II and their effect on L-Asparaginase activity was analyzed.

Effect of inducers: In order to determine the best inducer for enzyme production, various inducers namely, L-Leucine, L-Glutamine, L-Asparagine, L-Aspartic acid, Glycine, L-Alanine, L-Arginine were incorporated in 1 % w/v in the BSS-II and their effect on L-Asparaginase activity was analyzed.

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Detail procedure of Statistical method to optimize the L-Asparaginase enzyme production using solid state fermentation (SSF):
Amongst the variables screened using OFAT, glycerol, L-Asparagine and tryptone were identified as most influencing variables in L-Asparaginase production with the model organism. The combined effect of three independent variables glycerol, L-Asparagine and tryptone for production of L-Asparaginase was studied by using RSM. To examine the interactive effect of these factors, a central composite design (CCD) was employed within a range of –1 0 +1 in relation to production of L-Asparaginase as shown in **Table 2**.

Table 2: RSM factors and Level: response surface methodology

Factor	Component	+1	0	-1
A	Glycerol	0.50	1.00	1.50
B	L-Asparagine	0.50	1.00	1.50
C	Tryptone	0.25	0.50	0.75

The Design-Expert Version 6.0.8 Software (Stat-Ease, Inc. Minneapolis) was used in the study. Model fitting and graphical analyses were carried out using DE software. In this study, the experimental plan consisted of 20 trial runs. Model statistics computed were F-value, degree of freedom (DF), lack of fit, coefficient of determination (R2), and coefficient of variation (CV). Empirical model was generated both in terms of coded factors (standardized equation) and actual factors (unstandardized equation).

Final Equation in Terms of Coded Factors:
Enzyme activity = $-1.07+2.96 * A+2.52 * B -1.75 * C -1.35 * A^2-1.05 * B^2 +2.35 * C^2 -0.29 * A * B -0.60 * A * C +0.14 * B * C$
And in actual parameters units is given in the equation.

Final Equation in actual terms:
L-Asparginase Enzyme activity = -1.06991 +2.96145 * Glycerol +2.52145 * L-Asparagine -1.75309 * Tryptone -1.35273 * Glycerol^2 -1.05273 * L-Asparagine^2 +2.34909 * Tryptone^2-0.29000 * Glycerol * L-Asparagine -0.60000 * Glycerol * Tryptone +0.14000 * L-Asparagine * Tryptone.

Partial purification of the L-Asparaginase enzyme: To improve the activity of L-Asparaginase enzyme, the supernatant of the 5 gm lysed cell pellet (as mentioned in the above section) was subjected to ammonium sulphate precipitation at concentrations from 0 % to 80 % (21) Ammonium sulphate precipitate was dissolved in 0.01 M Phosphate buffer having pH 7 and analyzed for L-Asparaginase activity. The dialysis was performed using 0.01 M Phosphate buffer pH 7. The dialysed sample was filtered using 0.2 µ filter. Then, ultrafilration was performed using 50 cm2poyethersulphone membrane (molecular weight cut off (MWCO)-30 kDa) to concentrate the sample to 1/5 of the original volume. The retentate and permeate were analyzed for L-Asparaginase activity. Ammonium sulphate (g/L) to be added to the supernatant was calculated using the following formula:

$$g/L = [533(S_2-S_1)] / [100-(0.3*S_2)]$$

Where, g=grams of ammonium sulphate required; S1=% of initial saturation; S2=% of final saturation; the factor 533 represents the solubility of the salt in gram at 20 oC in 1 liter to make saturated solution.The experiments were performed in year, 2010-2011.

3. Results and Discussion

Different factors that affect the production of L-Asparaginase activity are substrate, seedculture media,seed age,moisture content, fermentation period, final enzyme extraction pH and time, impregnating medium, nitrogen and carbon sources.

Effect of seed culture media for L-Asparaginase enzyme production: To maximize the growth of *W.eutropha*, attempts were made by supplying the organism with the optimal mixture of small molecules. Experiments were performed using four different seed culture mediaincluding (a) TGY, (b) GPY, (c) Nutrient broth and (d) semi-synthetic. It can be seen that optical density and enzymatic activity are directly proportional to each other. But, TGY media supported good growth and enzyme production (2.18 ± 0.004 U/gds), which was not observed with any of the other media (GPY, nutrient broth and semi-synthetic media) tested (**Figure 1A**). Replacement of tryptone by peptone had no significant advantage, whereas cell growth and enzyme activity rigorously decreased.

Substrate screening for elemental content and L-Asparaginase enzyme production:
Four different agricultural solid substrates such as *Vigna radiate* bran, *Cajanuscajan*bran, *Glycine max* bran and *Cicerarietinum* bran were evaluated individually and in combination for their elemental content including carbon, nitrogen, hydrogen and protein content and for the biosynthesis of L-Asparaginase by *W.eutropha*. The nutrient composition of these substrates and the occurrence of L-Asparaginase activity in various individual brans and combinatorial brans are presented in **Table 1A-1B**. *Vigna radiate* bran was rich in all the three elements and the protein content whereas, the *Cicerarietinum*bran was with lowest elemental and protein content **(Table 1A)**. In terms of L-Asparaginase enzyme activity, *Vigna radiate* bran gave maximum enzyme activity (0.67 ± 0.0031U U/gds) followed by *Glycine max* bran and *Cajanuscajan* bran. Whereas, the *Cicerarietinum* bran showed lowest L-Asparaginase enzyme activity (0.33±0.0024) U/gds**(Table 1B)**. Study on an equal combination of substrate showed that combination of *Glycine max* bran, *Cajanuscajan* bran, and *Vigna radiate* bran showed almost equal amount of L-Asparaginase activity as that of shown by *Vigna radiate* bran alone **(Table 1B).** The combinations of *Vigna radiate* with *Glycine max* bran as substrate was second highest L-Asparaginase producer followed by *Vigna radiate* with *Cicerarietinum* bran. The combination of *Glycine max* bran with *Cajanuscajan* bran produced the lowest L-Asparaginase enzyme activity. *Vigna radiate* proved to be a good substrate for *W.eutropha* growth and L-Asparaginase enzyme activity. It is apparent from these results that *Vigna radiate* bran was the potent source of elemental nitrogen and carbon content which could support growth of *W. eutropha*. Looking towards these results, our study further envisaged on the parameters for the optimization of L-Asparaginase enzyme by *Vigna radiate*.

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Table 1: A- Showing nutrient, protein and enzyme activity of agricultural solid substrates; B-Showing combinatorial substrate combination and itsenzyme activity

A					
Substrate	Nutrient composition				Enzyme activity (U/gds)
	N (%)	C (%)	H (%)	Protein (mg/L)	
Vigna radiate bran	1.477	41.59	6.237	122.14	0.67±0.0031
Cajanuscajan bran	0.635	40.890	5.599	13.57	0.44±0.0018
Glycine max bran	1.295	40.103	5.862	125.00	0.63± 0.0034
Cicerarietinum bran	0.326	39.628	5.736	8.57	0.33±0.0024

B	
Substrate combination	Enzyme activity (U/gds)
Vigna radiate bran + Glycine max bran	0.48±0.0023
Glycine max bran + Cajanuscajan bran	0.32±0.0042
Cajanuscajan bran + Vigna radiate bran	0.39±0.0014
Vigna radiate bran + Glycine max bran + Cajanuscajan bran	0.65±0.0006

Optimization of seed age: Growth experiments were performed to determine the optimal time for cells harvesting. Figure 1B presents the data obtained at various time intervals from 8 hrs to 56 hrs respectively and showed that the enzyme production was related to phases of cell growth. With time, the activity rise has been observed and 48 hrs of seed age showed maximum L-Asparaginase activity of 0.65 ± 0.0056 U/gds whereas with further increase in the time no significant rise in enzyme activity was observed (**Figure 1B**). The results shown in figure 1B revealed that tryptone containing cultures has rapid motility and enzyme production during exponential phase and in the early stationary phase reduced activity has been observed. It may be inferred that cells are likely to undergo lysis during stationary phase which might resulted in reduced enzyme synthesis in the early stationary phase cell (**Figure 1B**). This result is in accordance with Albanese and Kafkewitz where Synthesis of L-Asparaginase enzyme by *Vibrio succinogenes* occurs throughout exponential phase (22).

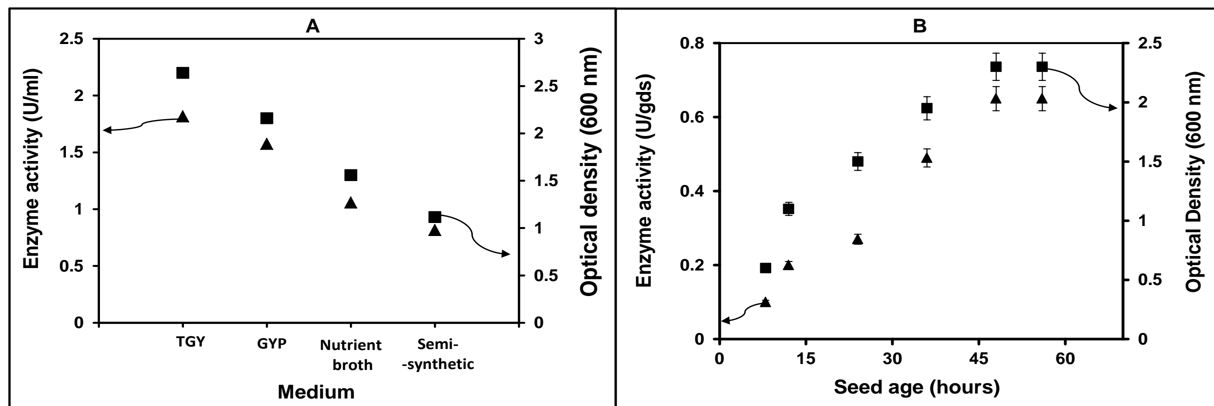

Figure 1: A- Showing seed/inoculums media development; B- Optimization of Seed Age

Optimization of initial moisture content and fermentation rate (period), pH as cell disruption tool and disruption time: Initial moisture content is an essential aspect of SSF since it directly affects enzyme production and its activity. Moisture makes oxygen availability to microbes and also helps to impregnate various additional nutrient components to the organism. Five different moisture contents were taken into account i.e. 5%, 10%, 15%, 20%, 25%, 30% and 40%. **Figure 2A** shows that maximum enzymes activity of 0.66 ± 0.004 U/gds was attained with 25% initial moisture content. This study also depicted decreased enzyme activity at low and high level of moisture content and did not prove to be efficient for *W. eutropha* growth and L-Asparaginase activity. However, the growth of bacteria and the maximum L-Asparaginase production with 25 % w/w moisture content is suitable to provide encourage and utilization of nutrients, as it showed significant cell mass and enzyme activity. This result also be attributed that higher and lower moisture level (other than 25%) decreases porosity, promotes development of stickiness, and increases the chances of contamination (4).

To determine the optimal incubation/fermentation time for the production of L-Asparaginase by *W.eutropha*, fermentation flasks were incubated for duration of 24, 36, 48, 72 and 96 hours. The analysis of enzyme activity at different time intervals showed that maximum activity of L-Asparaginase*i.e.*, 0.68 ± 0.006 U/gds was achieved with fermentation period of 72 hours (**Figure 2B**). With the further increase in the fermentation period of 96 hours, sharp decline in the enzyme activity and cell mass was observed. Thus, it was seen that the enzyme activity was increasing in the exponential phase of the growth curve. When incubation period was increased up to 96 hours, enzyme activity was decreased. The literature reports also specify that up to a certain limit, the fermentation period is directly proportional to the production of enzymes and other metabolites (23). Therefore, from the present study it is clear that at longer incubation periods, the enzyme activity decreased which might be due to depletion of nutrients, accumulation of toxic end products and change in the pH of the medium or loss of moisture.

The pH has marked effect on the type and amount of enzyme produced by a microbe. One organism may secrete different amounts and different types of enzymes depending upon the pH and composition of medium (24) Different organisms have different optimum pH and any changes in their optimum pH could result in a decrease in their enzymatic activity (25) L-Asparaginase enzyme being alkali soluble (Allisonet al., 1971, 26), the technique that was followed was optimization of alkaline pH as cell disruption tool. L-Asparaginase enzyme was extracted in soluble form in the aqueous phase in alkaline solution. The pH studied was ranged from 7.0 to 12.0 and the enzyme production was

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found to be maximum *i.e.*, 0.67 ± 0.002 U/gds when pH of the diluent was adjusted at pH 8.5 (**Figure 2C**). Microbial enzymes are produced in higher yield at pH near the maximal for enzyme production. In the present study, it was observed that the enzyme activity decreased significantly with increase in alkaline pH and the enzyme activity was maximum at pH 8.5 for the *W. eutropha*. The optimum pH value was varied for different microbes employed in L-asparaginase production. Mishra, 2006 has reported the maximum enzyme yield at pH 6.5 using agricultural waste by *Aspergillusniger*(**11**). The optimum of L-asparaginase obtained by *Thermoactinomyces vulgaris*pH 8-8.2 (**27**). So, individual enzyme acts best in a certain pH range which is particular to it and its growth and activities slow down with any appreciable increase or decrease in that value (28).

W. eutropha was stable at neutral pH with increase in the pH thus it has been proposed that there might have been physiological changes in the cell membrane proteins causing degradation of cell wall and loss of cell wall integrity due to physiological imbalance. It was observed that at cell disruption pH 8.5 optimal L-Asparaginase enzyme activity of 0.67 ± 0.002 U/gds was obtained. At lower and higher pH values used for cell lysis the activity had no significant effects (**Figure 2C**).

Further, pH as an optimized factor was incorporated in the study and time as a parameter was taken into account to obtain maximum enzyme extraction.Effect of cell disruption time for L-asparaginase was determined by incubation of the reaction mixture for a different period of time (15, 30, 45, and 60 minutes) after which, enzyme activity was determined. As **Figure 2D** shows, the optimum reaction time for L-asparaginase activity was 30 minutes (0.66 ± 0.004 U/gds). After this period, the enzyme activity did not increase significantly and was stable with an increase in cell disruption time.

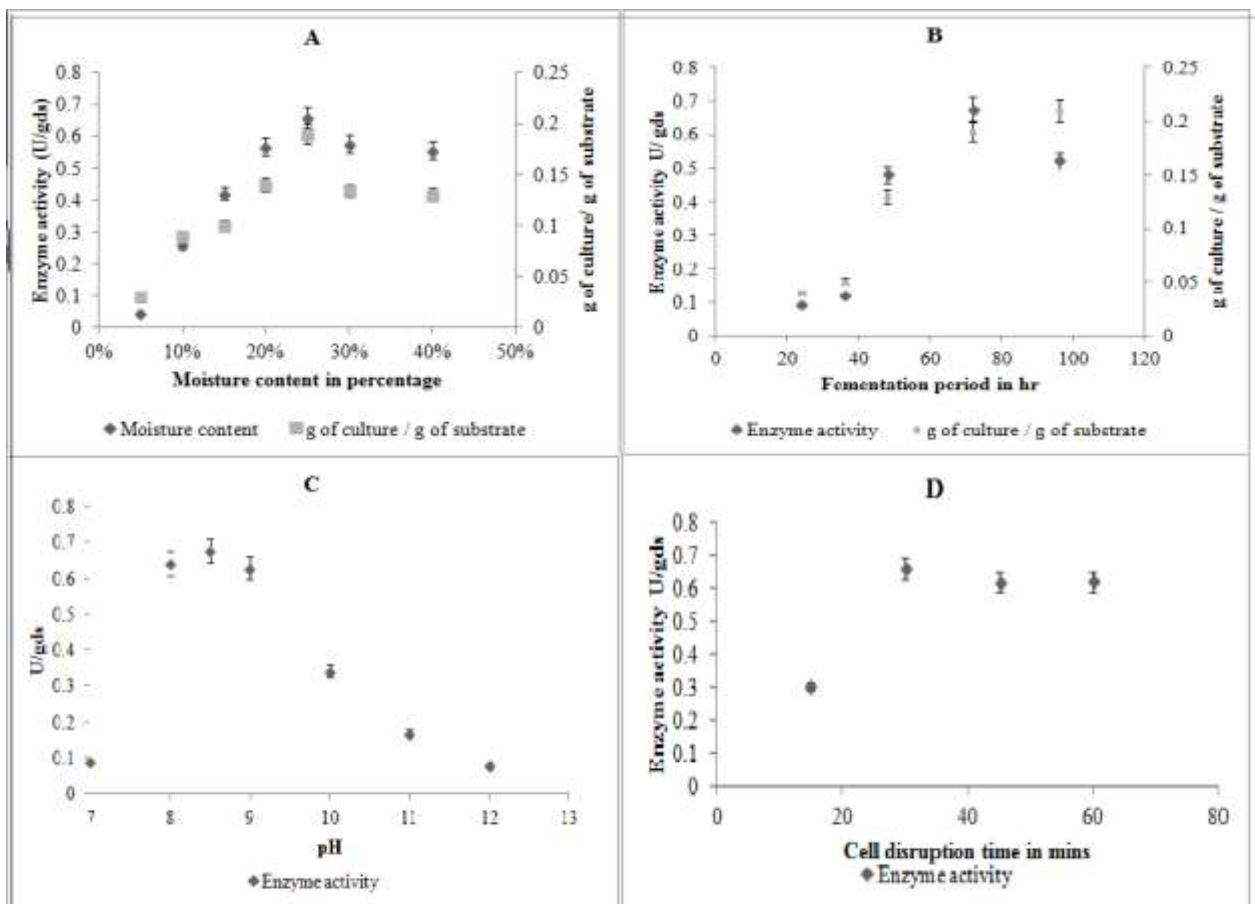

Figure 2: A-Showing percentage of initial moisture content; B- Effect of fermentation period; C- Effect of cell disruption solution pH; D- Effect of cell disruption time

Effect of nutrient media as diluents and inoculum volume on L-Asparaginase activity: The production of L-Asparaginaseby *W. eutropha*was investigated using different diluents for moistening the substrate. Composition of medium used for adjusting the moisture content and to provide additional nutrients for the bacterial growth included (a) Basal salt aqueous solution I (BSS-I) (b) Basal Salt aqueous solution II (BSS-II), (c) Basal Salt aqueous solution III (BSS-III) and distilled water as control. The production of enzyme was maximum *i.e.*, 0.714 ±0.003 U/gds when substrate was moistened with diluent BSS-II. Other diluents such as BSS-I and BSS-III gave enzyme production of 0.664 U/gds and 0.493 U/gds respectively (Figure 4A). The diluent BSS-IIconsisted of $(NH_4)_2NO_3$ and $MgSO_4$ $7.H_2O$ whereas, diluent BSS-III supplied additional nutrients such as of Na_2HPO_4, KH_2PO_4, NaCl and malt extract for the growth of an organism and enzyme production. Inspite of this, diluent BSS-III gave enzyme production of 0.493 U/gds. However, it can be inferred that additional nutrients present in other diluents have an inhibitory action on the growth of the organism and subsequently on the enzyme production, so gave less production of the enzyme (29).t can also be inferred

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that the nitrate containing BSS was most suitable and the phosphate containing BSS proved to be inhibitor to the activity of L-Asparaginase(**Figure 3A**).

Another important parameter for SSF is inoculum volume. Optimization of inoculum volume in SSF is necessary due to certain reasons. Inadequate volume could influence the mycelium growth, spore and biomass formation thereby leading to insufficient production of metabolites and end products (30). In this study, the effect of inoculums volume on L-Asparaginase production was studied with different inoculum levels. The substrate was inoculated with culture of 1-7 ml of inoculum level. The substrate was incubated for 5days i.e. 120 hr. After completion of fermentation, the enzyme was extracted and analyzed for the enzyme activity. The fungal growth was evidently observed more in the media inoculated with higher inoculum size. However, with respect to biomass and enzyme activity, higher yield was obtained with inoculum volume of 5 ml which produced 0.826 ± 0.002 U/gds of L-Asparaginase activity at an optical density of 2.3 (600 nm: 12,000 CFU/ ml) at 48 hours (Figure 3B). Whereas, low and high levels of inoculum volume other than 5 ml was found to inhibit the production of L-Apraginase might be due to induced product formation by insufficient biomass or non-volatile self-inhibiting substances formation at higher inoculum volume (6-7 ml) (31).

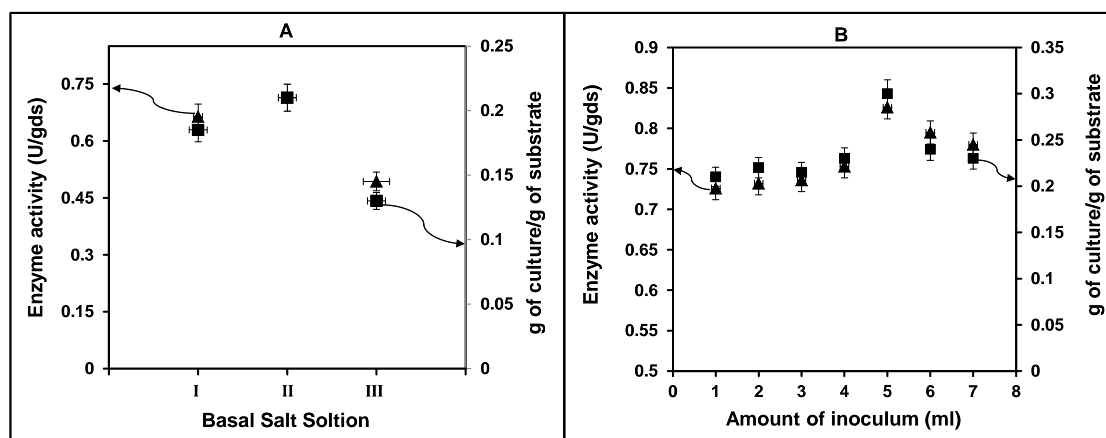

Figure 3: A-Effect of Basal salt solution and B: Effect of inoculums amount on enzyme activity

Effect of carbon, complex nitrogen sources and inducer on L-Asparaginase activity: Carbon sources are used for the energy and growth of microorganism during fermentation and subsequently for higher enzyme production. High titers of enzyme activity can be obtained in a medium rich of carbon source. However, in order to investigate the effect of carbon sources on L-asparaginase yield, different carbon sources were tested which were included in BSS-II including glucose, sorbital, glycerol, fructose, sucrose and lactose. Each of them at a concentration of 1% w/v with other optimized conditions was supplemented to the production medium (BSS-II) of *W. eutropha*. The results are presented in **Figure 4A**. As compared to other carbon sources tested, L-asparaginase production was high in the BSS medium containing glycerol. The data revealed that the maximum enzyme activity of 1.206 U/ml.gdm was obtained with glycerol and the least activity was obtained with fructose (0.063 U/ml.gdm). In accordance with Karthikeyan et. al (2014), glycerol as a carbon source exerted a considerable effect on L-asparaginase production by *Trichodermaviride* (32). From the above studies it is clear that glycerol is the best carbon source for L-asparaginase production by *W. eutropha.*

Next to carbon, complex organic nitrogen has pronounced effect on enzyme production. L-asparaginase production is under nitrogen regulation, therefore it requires supplementary nitrogen source in addition to the substrate. The presence of additional nitrogen sources along with nitrogenous compounds present in the substrate promotes significant growth and consequent enzyme production (33). Initially, the study was conducted to check the ability of *W. eutropha* to utilize various organic nitrogen sources. The organic nitrogen sources including Tryptone, Peptone, Malt extract and Yeast extract were incorporated at a concentration of 1 % w/v to production medium (BSS-II). Results showed that the microorganism was able to grow on all nitrogen sources tested and the enzyme production varied with different nitrogen compounds tested. Among the various nitrogen sources screened, in comparison to control with no nitrogen source tryptone gave appreciable results than Peptone, Malt extract and Yeast extract (**Figure 4B**). Maximum activity of 1.38 U/ml.gdm was obtained for Tryptone and the least activity of 0.486 U/ml.gdm for peptone was obtained. There has been much different response towards utilization of different nitrogen sources by variousfungal and bacterial isolates(34), reported malt extract as the best nitrogen source for maximum L-Asparaginase production (8.14 IU). An important observation has been made with respect to nitrogen uptake by many fungi that nitrogen uptake from $(NO_3)^{2-}$ media was markedly higher than other media. This may be due to the reason that in the presence of ammonium nitrate, the fungi can utilize both $(NH_4)^{2+}$&$(NO_3)^{2-}$ as nitrogen source (35). Additionally, a bacterial species, *Erwiniacarotovara*showed marked stimulation of the enzyme production with Tryptone and yeast extract (36). Therefore, it can be deduced that enzyme production in the organism is associated with nitrogen source which is most essential for growth as well as the enzyme production by the organism (37). From the above studies it is clear that tryptone can be used as the best organic nitrogen source for L-asparaginase production by *W. eutropha*.

L-asparaginase enzyme production is inducible and an ammonium ion plays an important role as inducer (1). Study

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was conducted to determine the effect of inducers incorporation in the optimized fermentation medium for the production of L-asparaginase enzyme. Various inducers including L-Leucine, L-Arginine, L-Aspartic acid, Glycine, L-Alanine, L-Glutamine and L-Asparagine were individually included in BSS-II with a concentration of 1 % w/v. The uncharged amino acid containing the terminal amide group in inducer L-Asparagine resulted in the highest titer of 1.092 U/ml.gdm followed by 0.665 U/ml.gdm by L-Glutamine. Remaining inducer showed no significant results in L-Asparaginase activity improvement compared to L-Asparagine as shown in **Figure 4C.** The greatest enzyme activity found when L-asparagine is used possibly due to its inducer nature which highly stimulated L-asparaginase production. Thus, as an inducer, L- Asparagine in the media proved to be an efficient stimulant to enhance the production of the secondary metabolite L-Asparaginase enzyme from *W. eutropha*.

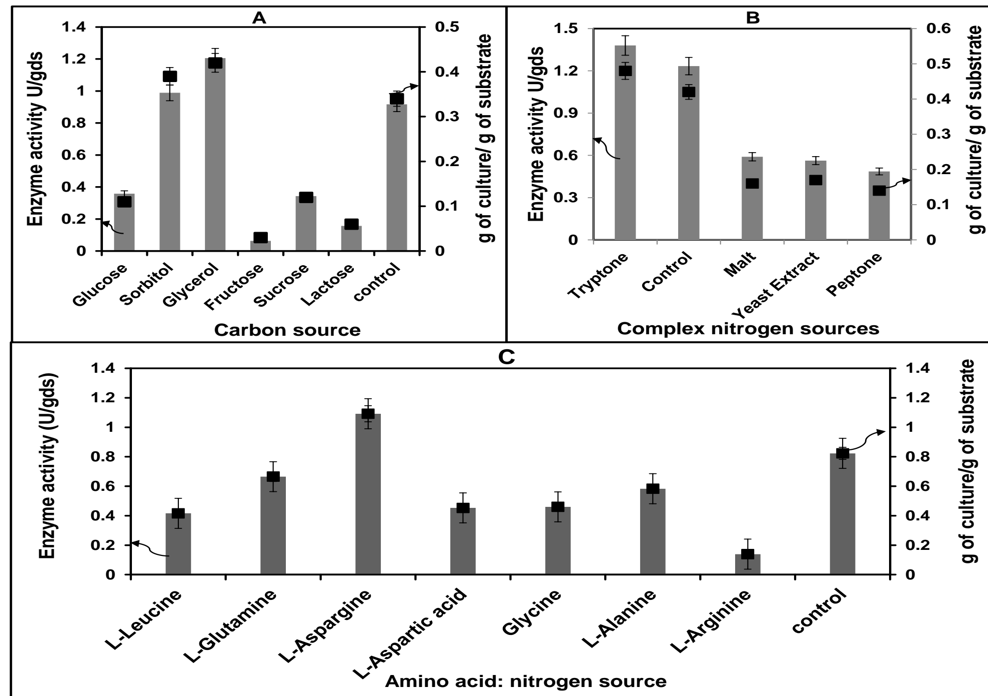

Figure 4: A- Showing effect of different carbon sources; B- Showing effect of different complex organic nitrogen sources; C- Showing effect of different inducers/nitrogen sources on enzyme activity

Statistical optimization of the selected medium factors by RSM:

After performing the first optimization with OFAT, the significant factors for L-asparaginase production from *W.eutropha* were identified as glycerol, tyrptone and L-asparagine using Response surface, Central composite quadratic model (Table 3). The correlative effect of three variables that had an effect on L-Asparaginase production were investigated by statistical optimization using RSM, in order to find the optimum conditions to increase the enzyme content of the fermented product using*W.eutropha* as a model organism. Among the variables screened, glycerol, tyrptone and L-asparagine were identified as most potent significant variables in L-asparaginase production. The combined effect of these three independent variables (glycerol, tyrptone and L-asparagine) were examined for their combined effect using a central composite design (CCD) which consisted of 20 experimental trials and is shown in Table 3.

The final model equation in terms of coded equation (equation 1) and in actual terms (equation 2) is shown as:

Enzyme activity = $-1.07+2.96*A+2.52*B-1.75*C-1.35*A^2-1.05*B^2+2.35*C^2-0.29*A*B-0.60*A*C+0.14*B*C$------------------(Equation 1)

L-Asparginase Enzyme activity = $-1.06991 +2.96145 * Glycerol +2.52145 * L\text{-}Asparagine -1.75309 * Tryptone -1.35273 * Glycerol^2 -1.05273 * L\text{-}Asparagine^2 +2.34909 * Tryptone^2-0.29000 * Glycerol * L\text{-}Asparagine -0.60000 * Glycerol * Tryptone +0.14000 * L\text{-}Asparagine * Tryptone$--------------------(Equation 2)

The run 14 showed maximum L-Asparaginase production of 1.42 U/gds. Optimization with RSM study confirmed for maximum L-Asparaginase activity from *W.eutropha* at the optimum concentration of glycerol 1.00 % w/w, L-Asparagine 1.00 % w/w and Tryptone 0.25 % w/w in BSS –II.

Table 3: The RSM design

Run	A: Glycerol (%w/w)	B: L-Asparaginase (%w/w)	C: Tryptone (%w/w)	Enzyme activity (U/gds)	
				Experimental	Predicted
1	0.50	0.50	0.75	0.85	0.84
2	1.50	1.50	0.25	0.6	0.59
3	0.50	1.50	0.25	0.9	0.87
4	1.50	0.50	0.25	0.61	0.6
5	0.50	1.50	0.75	1.2	1.1
6	1.50	1.50	0.75	0.6	0.62
7	1.00	1.00	0.75	1.3	1.4
8	0.50	0.50	0.25	0.62	0.62
9	1.00	1.00	0.50	1.2	1.23
10	1.00	0.50	0.50	0.8	0.81
11	1.00	1.50	0.50	1.1	1.16
12	1.50	0.50	0.75	0.54	0.55

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13	1.50	1.00	0.50	0.65	0.67
14	1.00	1.00	0.25	1.42	1.45
15	1.00	1.00	0.50	1.2	1.23
16	1.00	1.00	0.50	1.1	1.1
17	1.00	1.00	0.50	1.33	1.35
18	1.00	1.00	0.50	1.2	1.3
19	1.00	1.00	0.50	1.1	1.16
20	0.50	1.00	0.50	1.1	1.13

The statistical significance of the model equation was checked using F-test Analysis of Variance (ANOVA). The fitness of the models was also expressed by the coefficient of determination, R^2, for the quadratic model, which was found to be 0.9523 for the enzyme production. This value indicates that there was 95.23% of response variability in enzyme production.

Table 4: represents the effect of each variable along the mean squares, F-values and p-values.

ANOVA for Response Surface Quadratic Model, Analysis of variance table [Partial sum of squares]						
Source	Sum of Squares	DF	Mean Square	F value	Prob> F	
Model	1.51	9	0.17	22.2	< 0.0001	significant
A	0.32	1	0.32	42.04	< 0.0001	
B	0.23	1	0.23	30.48	0.0003	
C	0.028	1	0.028	3.68	0.0839	
A2	0.31	1	0.31	41.72	< 0.0001	
B2	0.19	1	0.19	25.27	0.0005	
C2	0.059	1	0.059	7.86	0.0187	
AB	0.042	1	0.042	5.58	0.0398	
AC	0.045	1	0.045	5.97	0.0347	
BC	2.45E-03	1	2.45E-03	0.33	0.5812	
Residual	0.075	10	7.54E-03			
Lack of Fit	0.039	5	7.86E-03	1.09	0.4638	not significant
Pure Error	0.036	5	7.22E-03			
Total	1.58	19				

Table 4: Analysis of Variance

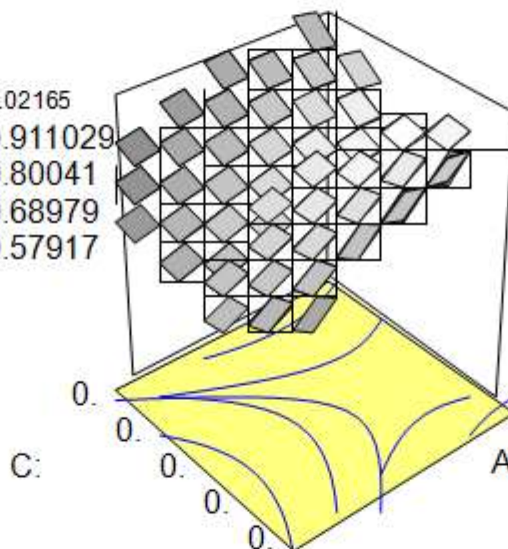
The model F-value of 22.20 implies the model is significant. There is only 0.01% chance that a "Model F-value" this large could occur due to noise. RSM performed showed the coefficient of determination (R^2) of the model as 0.9523, indicating that the model adequately represented the real relationship between the parameters chosen. The results of the error analysis indicated that the lack of fit was insignificant. The coefficient of variation of 8.94 indicated that the model is reproducible.

To determine the optimal levels and the interaction effects between the process variables, the three dimensional surface plots were constructed. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. The fitted response for the regression model was plotted in figure 6 (A-C). 3D graphs generated for the pair-wise interaction of the three factors explain the role played by factors affecting L-Asparaginase production.

DESIGN-EXPERT

Enzyme activity
X = A: Glycerol
Y = C: Tryptone
Actual

B: L-Asparagine =



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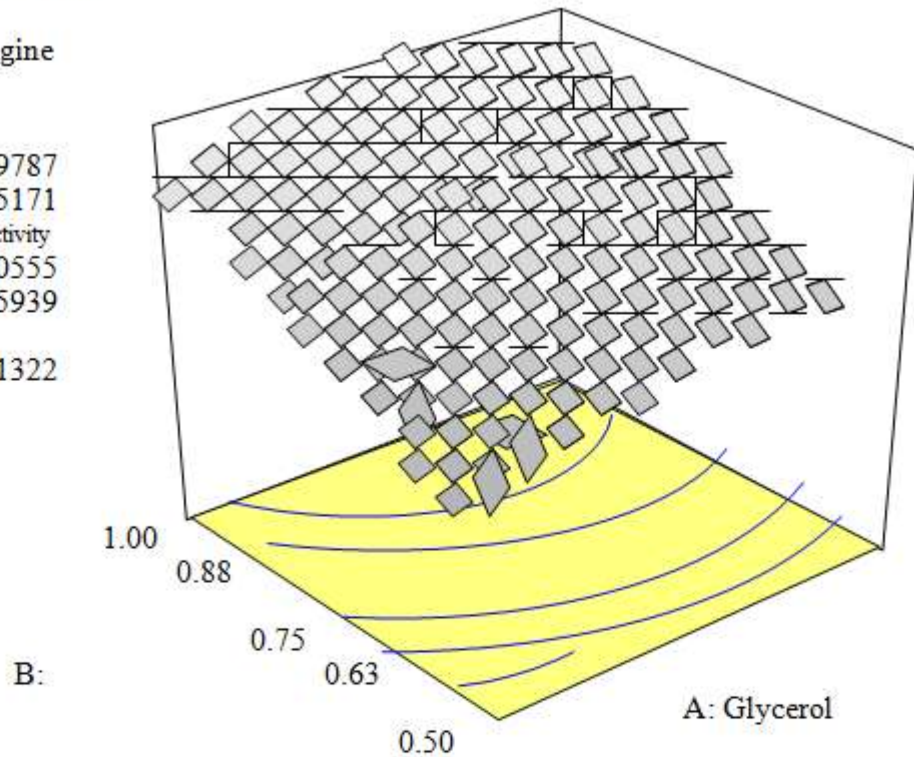

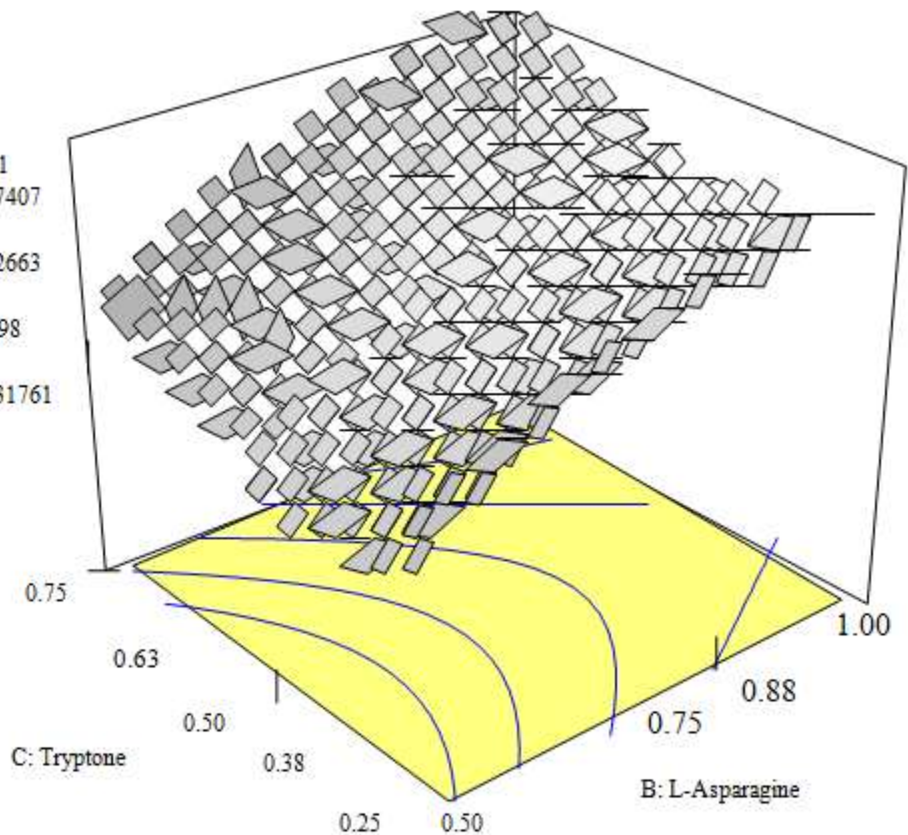

Figure 5: (a) A: Tryptone and C: Glycerol interaction; (b) C: Glycerol and B: L-Asparagine interaction; (c) B: L-asparagine and A: Glycerol interaction

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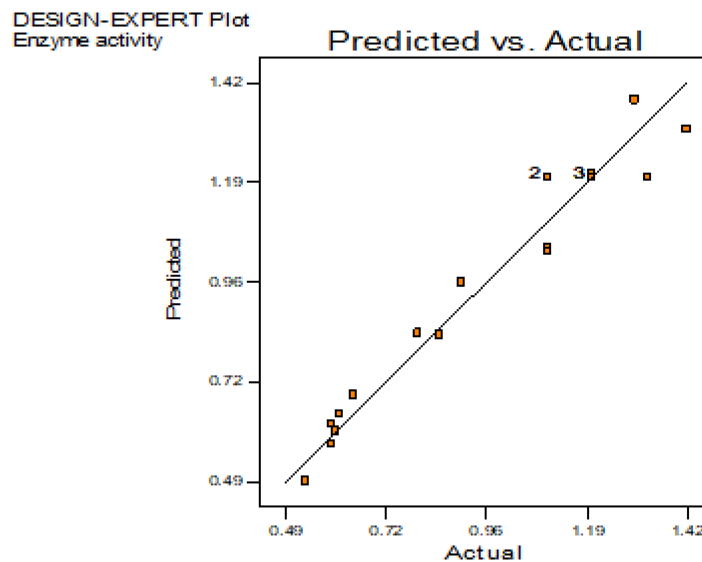

Figure 6: Parity plot showing the distribution of experimental verses predicted values.

The results obtained were fitted in the quadratic second order polynomial to explain the dependence of L-Asparaginase biosynthesis on media components.

Enzyme purification by Ammonium sulphate precipitation: Isolation of L-asparaginase was most effective with ammonium sulphate precipitation. In order to evaluate the effect of ammonium sulphate concentration on the fermentation medium, SSF was carried out with different fractions of ammonium sulphate varying from crude 0%, 20-40 %, 40-60 % and 60-80 % (w/v). As shown in Table 5, stepwise precipitation of the enzyme at 0, 20-40, 40-60 and 60-80% saturation gave highest purification at 60-80% saturation (percentage recovery of 30.60 % with 10 ml of supernatant). On the other hand, yield of the protein and total protein for 60-80% fraction was, however, less compared to that obtained with other ammonium sulphate fractionation used in the study.

Table 5: Ammonium sulphate precipitation of L-asparaginase produced by *W. eutropha* by SSF

Parameters of *Vigna radiate* bran	Sample $(NH_4)_2SO_4$ fractionation (w/v)			
	Crude 0 %	20-40 %	40-60 %	60-80 %
ml of supernatant	50	6	8	10
Enzyme activity (U/gds)	5.62	5.96	6.63	8.6
Total Activity (U)	281	35.76	53.04	86
Protein (mg/ml)	0.85	0.65	0.53	0.38
Total Protein (mg/ml)	42.5	3.9	4.24	3.83
Specific activity (U/mg)	6.61	9.16	12.0	22.45
Purification fold	1	1.38	1.81	3.39
% Recovery	100	12.72	18.12	30.60

Microfiltration was then performed of ammonium sulphate precipitated fractions dissolved in phosphate buffer (pH 7) obtained after dialysis. Microfiltration extract showed the enzyme activity 7.1 U/ml, total activity 355 U, protein 0.68 mg/ml, total protein 34 mg and specific activity 10.44 U/mg. The ultra filtrationretentate produced the enzyme activity of 32.527 U/ml, total enzyme activity of 325.27 U, protein of 0.54 mg/ml and total protein of 5.4 mg and specific activity of 60.23 U/mg, fold purity 5.76 and percentage recovery 91.62 %.

4. Conclusions

Solid state fermentation using pretreated *Vigna radiate* bran proved to be one of the easy, economic and an alternate method to submerged fermentation for L-Asparaginase enzyme biosynthesis from *Wautersiaeutropha*NRRL B-2804. The pretreated solid agricultural waste material could be used as nutrition and encourage for bacterial growth. Modifying various media parameters, by addition of various components to basal salt solutions have proved to be significant for improving the L-Asparaginase biosynthesis. Suitable moisture is required for the bacteria to propagate on the solid agricultural waste. The L-Asparaginase enzyme is produced in the exponential phase of the bacterial growth curve and is directly related to the biomass production. Nitrogen sources, carbon sources along with the BSS have shown to cause significant improvements in L-Asparaginase activity. The amino acid L-Asparagine have proved to be and inducer/ precursor for L-Asparaginase enzyme biosynthesis. Processes such as ammonium sulphate precipitation, microfiltration and ultra filtration have further improved the enzyme activity. Further studies should be taken to increase the yield and purity of the L-Asparaginase enzymes.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, SadhanaSathaye. and YogitaLabrath.; Methodology, SadhanaSathaye and YogitaLabrath.; Software, SadhanaSathaye and YogitaLabrath.; Validation, VanitaNimje., UjwalaGosawi. and SadhanaSathaye.; Formal Analysis, YogitaLabrath.; Investigation, SadhanaSathaye.; Resources, SadhanaSathaye and YogitaLabrath.; Data Curation, YogitaLabrath.; Writing-Original YogitaLabrathPreparation, YogitaLabrath.; Writing-Review & Editing, YogitaLabrath, SadhanaSathaye, VanitaNimje, UjwalaGosavi.; Visualization, UjvalaGosavi, SadhanaSathaye.; Supervision, SadhanaSathaye.; Project Administration, SsdhanaSathaye.; Funding Acquisition, SadhanaSathaye and YogitaLabrath",

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Conflicts of Interest: **"The authors declare no conflict of interest."**

Appendix A

Abbreviations: gds: gram of dry substrate. P.F.: purification factor, RSM (Response Surface Methodology, ALL: Acute Lymphoblastic Leukemia, ASCR: American society on cancer research, SmF: submerged fermentation. SSF: Solid state fermentation, NRRL: National Regional Research Laboratory, MWCO: Molecular weight cut off. TGY: Tryptone, glucose and yeast extract, GPY: Glucose, peptone and yeast extract, E.C. no.: Enzyme commission number, BSS: basal salt solution, rpm: rotation per minute.

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