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A model for finding new L-asparaginase producing microorganisms using Taguchi design of experiments

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Abstract

The current study suggests the screening of soil samples for L-asparaginase producing microorganisms and optimization of enzyme production by Taguchi Design of Experiments as a suitable model for finding alternate L-asparaginase enzyme to the ones used in current therapeutic methods. A bacterium designated as strain MH01 was isolated that could effectively breakdown L-asparagine. It was found to be specie of genus *Bacillus* based on morphological, biochemical analysis and 16S rRNA gene sequencing, resulting in 96% similarity with *Bacillus marcorestinctum* strain LQQ (GQ900516). Enzyme production was statistically optimized using Taguchi design of experiment. The maximum specific activity 0.8 IU/µg was obtained at 30°C temperature, 2:1 inoculum to substrate ratio and a pH 5.0. L-asparaginase produced under optimized conditions was partially purified by size exclusion column chromatography using sephadex G-100. The molecular weight was estimated to be 41.88 kDa. The enzyme was purified 1.79 folds with 31.98% yield and 1.586 IU/µg specific activity with an optimum temperature of 30°C and pH of 8.0-8.5. The enzyme had K_m value of 0.389 mM, V_{max} value of 30395 μML^{-1} min^{-1} and K_{cat} value of 429.36 s^{-1} which indicates its efficiency and potential for further study and proving the success of our model in finding an alternate L-asparaginase enzyme.

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Introduction

L-asparaginase (EC 3.5.1.1), an amidohydrolase enzyme, catalyzes the breakdown of L-asparagine yielding L- aspartic acid and ammonia [1]. L-asparagine is a non-essential amino acid which is present in sufficient quantities in blood and can be synthesized by the body. It was found that to keep up the protein synthesis during rapid cell proliferation by cancerous cells, aberrantly large amounts of L-asparaginase in blood is required [2]. The enzyme, L-asparaginase, depletes the concentration of this amino acid resulting in remission in cancerous cells, whereas normal cells can still produce L-asparagine in sufficient quantities suiting to their metabolic needs using the gene L-asparagine synthetase [3]. L-asparaginase which is currently used for chemotherapy is isolated from two sources: *Escherichia coli* and *Erwinia* species. Patients undergoing therapy using the enzyme isolated from these bacteria may exhibit immunogenic response, such as anaphylaxis, with the former showing stronger immunogenic response than the latter [4]. Furthermore, L-asparaginase from these sources also exhibit glutaminase activity which was previously thought to hinder its medical application. However, a recent study found that glutaminase activity has a link with tumor regression in certain types of cancer, while in others it is unimportant [5].

Two types of resistance which occurs in L-asparaginase therapy are the immergence of specific antibodies against the enzyme and the de-repression of L-asparaginase synthetase gene, whereas toxicity ranges from acute hypersensitivity, hyperglycemia to hepatocellular dysfunction or pancreatitis [2]. To overcome such complications modified L-asparaginases were introduced, such as PEGylated L-asparaginase which increased its stability, however the emergence of PEG specific antibodies resulted in the rapid removal of the enzyme. Another method, entrapment in Red Blood Cells was used which resulted in increased stability but with no significant effect on immunogenicity [6, 7]. Similarly, recombinant L-asparaginase from other sources expressed in *E. coli* to decrease such problems have also been reported and seem promising but are not definitive per current studies, thus necessitating further investigation into finding an ideal enzyme this way with low antigenicity and toxicity whilst having higher efficacy. Such ideal enzyme should also have increased stability, decreased glutaminase activity and be easily available.

Recently, the discovery of acrylamide in foods raised a serious concern [8]. Acrylamide, a potent carcinogen was shown to be present in various foods, such as baby food, biscuits, French fries, and even in roasted coffee [8]. L-asparagine is a non-essential amino acid and is present in most food types, which when are processed at a high temperature, increase the chance of acrylamide formation due to the millard reaction between free asparagine amino acid and the different reducing sugars present. The underlying mechanism of acrylamide formation is an intricate, multi-step process which takes place usually at elevated temperatures [8-10]. Although processing of different food types results in different concentrations of acrylamide, its presence altogether is a serious concern and various strategies need to be employed to mitigate its formation. One such strategy is the use of L-asparaginase enzyme to deplete the free L-asparagine stock in the medium, rendering it incapable to produce acrylamide [11]. Another strategy is the fermentation of such foods to reduce their free L-asparagine content. However, fermentation of food alters the chemical properties, such as sensorial, texture and appearance which may not be desirable. The usage of L-asparaginase reduces free L-asparagine and does not alter the properties of the food preserving it in its original form [8]. The current study focuses on screening of environmental samples for the isolation of novel L-asparaginase producing microbial strains followed by optimization of enzyme production using Taguchi method as an effective model in finding alternate L-asparaginase producing microorganisms. Using this model, a new L-asparaginase producing *Bacillus* sp. has been isolated from soil with moderate glutaminase activity. The enzyme production was optimized followed by partial purification and characterization.

Methods

Isolation of L-asparaginase producing microorganisms

L-asparaginase hydrolyses asparagine into aspartic acid and ammonia. The production of the latter can raise the pH of the medium and can be detected by a suitable indicator. Modified Czapek Dox's medium supplemented with L-asparagine and an indicator bromothymol blue (BTB) was used for isolation of L-asparaginase producing microorganisms [12]. The composition of the medium (g/L): trace $CuNO_3$, trace $FeSO_4$, 0.52 KCl, and 1.52 KH_2PO_4 $MgSO_4.7H_2O$, trace $ZnSO_4.7H_2O$, 2.0 Glucose, 10.0 L-asparagine and 20.0 Agar. Soil sample was collected from potato fields in Kasur district, the Punjab, Pakistan. 10g of soil was stirred in 100 mL of phosphate buffer, subsequently serially diluted and aseptically inoculated on modified Czapek Dox's agar plates. L-asparaginase producing colonies which produce blue zones were purified on Nutrient Agar plates and subsequently tested for enzyme activity. Each isolate's L-asparaginase producing capacity was be tested in the production broth at similar conditions (as mentioned below) and the isolate which produced the highest enzyme activity was selected for further study.

L-asparaginase production broth

Overnight cultures of L-asparaginase producing bacteria were inoculated in production broth which is composed of modified Czapek Dox's media supplemented with 10 g/L L-asparagine amino acid substrate. The production broth is incubated at 37 °C for 24 hours or as specified. Furthermore, cell suspension was centrifuged and the enzyme containing supernatant was re-suspended in 50 mM Tris buffer (pH 8.6).

L-asparaginase activity

L-asparaginase activity was determined by using modified version of Imada *et al.* [13]. 100 µL of enzyme solution was reacted with 0.9 mM L-asparaginase for one hour at 37 °C. Special care should be taken that both solutions are equilibrated at 37°C prior to incubation. The reaction was stopped by addition of 1.5 M trichloroacetic acid and subjected to direct nesslerization and finally absorption was measured at 436 nm. Ammonia concentration was determined by the ammonia standard curve constructed by nesslerization of different concentrations of ammonium sulfate prepared from 6 mM stock solution followed by measuring absorption at 436 nm. The amount of ammonia produced was used to calculate units of L-asparaginase where one unit corresponds amount of enzyme required to liberate 1 µmoles of ammonia per minute at standard temperature of 37°C. Additionally, estimation of protein concentration was done by Lowry method, using bovine serum albumin (BSA) as standard, which was further used to calculate specific activity [14].

Identification and phylogenic analysis of L-asparaginase producing bacterium

Strain MH01 was identified morphologically as well as biochemically according to Bergey's Manual of Determinative Bacteriology [15]. Molecular identification was carried out by sequencing of 16SrRNA gene. For this purpose, the DNA was extracted by DNA extraction kit (QIAGEN), and 16S rRNA gene sequence was amplified using 27F' (5'-AGAGTTTGATCCTGGCTCAG-3') and 1494R' (5'-CTACGGCTACCTTGTTACGA-3') bacterial primers. The amplified PCR product was sequenced by Macrogen Service Center (Geunchun-gu, Seoul, South Korea). The sequence obtained was computed for nearest relatives in the NCBI database using BLAST tool and homologues were analyzed for phylogeny using Molecular Evolutionary Genetic Analysis (MEGA) version 7 [16]. Phylogenetic tree was constructed based on the maximum likelihood method [17], and the sequence was submitted to NCBI GenBank.

Optimization of culture conditions using Taguchi Design of Experiments (DOE)

The Taguchi DOE finds control factors within process variability and natural environmental factors in an experimental setting [18]. In a Taguchi experiment, there are two designs; inner array and outer array. The control factors constitute the inner arrays while

the noise factors, such as environmental factors constitute the outer array. The Taguchi design analyzes the interaction between the two arrays generating a signal to noise ratio. The signal to noise ratio is a statistically derived value which depends on whether the goal is to minimize, maximize or match the quality characteristic target value. Furthermore, ANOVA was used to find significant factors along with their correlation with enzyme production [19, 20]. Factors to be optimized and their corresponding levels are summarized in Table 1. The Taguchi design, in Table 3, is L_{18} orthogonal array with "larger is better" formula created using SAS JMP 10™ which contains combinations of different levels of each factor

Partial purification of L-asparaginase from Strain MH01

Production under optimized condition

100 mL production broth was inoculated incubated at the optimized conditions determined through Taguchi DOE and was centrifuged after incubation, at 8,000×*g* for 10 min at 4°C (Kokusan Model H-2600) and cell free supernatant was collected for protein precipitation.

Ammonium sulfate precipitation

Culture free supernatant with L-asparaginase activity was subject to ammonium sulfate precipitation [$(NH_4)_2SO_4$]. Solid ammonium sulfate was added with gentle stirring at 4°C until the solution reached 20% saturation. The solution was centrifuged at 8000xg for 10 min and the supernatant was collected. Then, solid $(NH_4)_2SO_4$ was added to the supernatant until the solution reached 80% saturation. The solution was centrifuged, and precipitates were dissolved in 6 mL of 50 mM sodium citrate buffer (pH 8.6). Excessive salt was removed from crude protein through dialysis tubing and then stored in freezer at -80°C.

Column chromatography

About 2 mL of the crude enzyme extract was loaded on Sephadex G-100 (10/50 mm, GE Healthcare UK) with 0.02% Sodium azide. The enzyme was eluted using 50-mM tris buffer (pH 8.9) at a flow rate of 0.6 mL/min. A total of twenty fractions were collected and tested for activity and protein content. The active fractions were pooled and then concentrated by ultrafiltration (Millipore, USA).

Estimation of Molecular weight

The underlying principle of determining molecular size through gel filtration chromatography is same as stated above [21]. Different sized proteins were run through the column containing Sephadex G-100 gel (10/50 mm) where larger proteins pass through the column before smaller ones and are eluted in different fractions. A standard curve of log molecular weight and fraction number was constructed and used to estimate protein size by noting the fraction number in which the protein is eluted and its corresponding molecular weight [22]. The proteins used to construct a standard curve were anti-diuretic hormone, Bovine serum albumin, Myoglobulin, lysozyme, TyrGlyGly tripeptide, Azide and Lactoglobulin B.

Characterization of Partially Purified L-asparaginase

Effect of temperature and pH on enzyme activity

The effect of temperature on enzyme activity was studied over a temperature range of 10–70 °C, and then activity was determined under standard assay conditions. Similarly, the activity of enzyme was monitored at different pH ranges (2.0–12.0). The buffer systems (100 mM) used in this study were: sodium citrate (pH 3.0–6.0), sodium phosphate (pH 7.0), Tris-HCl (pH 8.0) and glycine NaOH (pH 9.0–12). Results were plotted in a graph with pH range or Temperature as x axis and enzyme activity (IU/mL) as y axis. Furthermore, error bars were added at 95% confidence interval (0.05 pvalue).

Effect of substrate concentration on enzyme activity

Effect of substrate concentration on L-asparaginase activity was checked by subjecting the enzyme to various concentration of L-asparagine produced from 189 mM stock that was diluted to get 1.89, 3.78, 5.68, 7.57, 9.47, 11.36 and 13.25 µM concentration.

Subsequently, a lineweaver burk plot was made using linear regression on the reciprocal of substrate concentration and activity. V*max* and K*m* of the enzyme were calculated by taking inverse of the y intercept and x intercept respectively.

Glutaminase activity

The presence of glutaminase activity is relevant for tumor regression in some type of cancer cells. Thus the presence of glutaminase activity was calculated by substituting 189 mM L-asparagine with 189 mM L-glutamine.

Statistical analysis

Taguchi designs were created and analyzed by using SAS JMP version 10 software, whereas all other analysis, including graphs, error bars and linear regression were done using Microsoft Excel 2016 at 95% confidence limit ($p<0.05$).

Results

Isolation of L-asparaginase producing microorganisms

L-asparaginase producing bacterial strains were isolated from soil samples of potato fields in District Kasur, Punjab, Pakistan. The presence of enzyme was indicated by change in color of medium from yellow to dark blue due to breakdown of L-asparagine into ammonia and aspartic acid. A total of 3 strains, identified as strong L-asparaginase producers based on the blue zone size were purified and inoculated into modified Czapek Dox's broth supplemented with L-asparagine for L-asparaginase production.

Table 1. Different factors and their assigned levels for optimizing L-asparaginase production.

Factor	Level 1	Level 2	Level 3
Inoculum size (0.5%)	0.5 OD	1.0 OD	—
pH	5	7	9
Substrate concentration (%)	0.25	0.5	1.0
Temperature (°C)	30	35	40

A bacterium, designated as strain MH01 was selected based on maximum enzyme production i.e., 13.43 IU/mL within 72 hours at 37°C and stored in nutrient agar slants for further studies.

Identification of L-asparaginase producing bacterial strain

Biochemical tests, summarized in Table 2 were performed to identify MH01 strain. It was found that MH01 is a gram positive, motile, endospore producing rod, which is catalase positive, suggesting it to be a member of the genus *Bacillus*. Similarly, a sequence of 1478 nucleotides of 16S rRNA was obtained and submitted to NCBI GenBank under the accession number KX225412. Furthermore, phylogenetic analysis confirmed strain MH01 to be a member of the genus *Bacillus* with 96% similarity to *Bacillus marcorestinctum* strain LQQ. Figure 1 show the phylogenetic tree based on maximum likelihood method placing MH01strain with its closely associated *Bacillus* species.

Optimization of culture conditions

Taguchi DOE was made in SAS JMP™ v10 using L_{18} orthogonal array. Protein concentration and enzyme activity of each reaction was determined to calculate specific activity which was put in the software to determine the individual contribution of each factor and the effect of factors in combination on enzyme production. It was found that reaction 1 and 17, in Table 3, showed the highest specific activity at 30°C and pH 5.0-9.0 (reaction 1 and 17), and 2:1 inoculum to substrate ratio in both case (0.5:0.25 and 1.0:0.25 for 1 and 17).

The reactions were compared based on enzyme activity, protein concentration and specific activity. Reaction 1 conditions were chosen as optimum as compared to 17 because of a higher predicted mean, 0.68 IU/µg than 0.58 IU/µg even though the specific activity was same i.e., 0.80048 and 0.80566 IU/µg, however reaction 1 showed higher protein concentration and activity (88 µg/mL and 71 IU/mL)

than reaction 17 (47 µg/mL and 38 IU/mL). Reaction 1 has lower substrate and inoculum requirements and exhibits more enzyme activity as compared to reaction 17. Furthermore, statistical analysis of the results attested the decision of choosing reaction 1 conditions as optimum.

Table 2. Results of biochemical tests performed on MH01 strain.

Biochemical tests	Results
Gram Staining	+
Shape	Rods
Endospore	+
Catalase	+
Oxidase	-
Urease	-
Citrate	+
Motility	Motile
Gelatin Hydrolysis test	+
⇨ Oxidative fermentation of Carbohydrate	
Carbohydrate	Result
Glucose	+
Sucrose	+
Xylose	-
Arabinose	-
Maltose	+

The impact of individual factors, shown in Figure 2 show the contribution of each levels (x axis) towards the specific activity (y axis) of the enzyme. Table 4 shows the effect of different factors represented by mean values of specific activity (IU/µg) on enzyme production.

Table 3. Taguchi Design of Experiments L_{18} orthogonal array created in SAS JMP version 10**.**

Sr.#	Inoculum size	pH	Substrate %	Temperature °C		Protein Conc µg	Enzyme activity IU	Specific activity U/µg	SN Ratio	Predicted mean
1	0.5 OD	5	0.25	30	−−−−	*88.95238*	*71.20464*	*0.80048*	-1.933	0.682492
2	0.5 OD	5	0.50	35	−−00	*303.3333*	*69.64646*	*0.229604*	-12.78	0.260035
3	0.5 OD	5	1	40	−−++	111.619	22.86308	0.204831	-13.772	0.141426
4	0.5 OD	7	0.25	30	−0−−	56.46349	24.19042	0.428426	-7.3625	0.523223
5	0.5 OD	7	0.50	35	−000	141.4286	22.79461	0.161174	-15.854	0.100766
6	0.5 OD	7	1	40	−0++	56.09524	0.796857	0.014205	-36.951	-0.01784
7	0.5 OD	9	0.25	35	−+−0	194.819	30.65095	0.15733	-16.064	0.160778
8	0.5 OD	9	0.50	40	−+0+	175.7143	21.67228	0.123338	-18.178	0.165662
9	0.5 OD	9	1	30	−++−	65.77143	25.15376	0.382442	-8.3487	0.485292
10	1 OD	5	0.25	40	+−−+	141.9492	40.23008	0.283412	-10.952	0.22931
11	1 OD	5	0.50	30	+−0−	131.5619	64.93752	0.493589	-6.1327	0.672432
12	1 OD	5	1	35	+−+0	451.2381	45.2413	0.10026	-19.977	0.126482
13	1 OD	7	0.25	35	+0−0	1142.381	84.84848	0.074273	-22.583	0.073673
14	1 OD	7	0.50	40	+00+	189.0476	8.653199	0.045773	-26.788	0.078557
15	1 OD	7	1	30	+0+−	48.7619	21.09989	0.432713	-7.276	0.398187
16	1 OD	9	0.25	40	++−+	255.7143	16.39731	0.064124	-23.86	0.138569
17	1 OD	9	0.50	30	++0−	*47.80952*	*38.51852*	*0.805666*	-1.8769	0.581691
18	1 OD	9	1	35	0	789.0476	27.48597	0.034834	-29.16	0.035742

The inoculum size brings the least amount of change in the specific activity as seen by the difference (0.018576) in the mean values of 0.277981 and 0.259405 of 0.5 and 1.0 OD. Furthermore, acidic pH contributes more towards specific enzyme activity than neutral and basic pH, additionally it is observed that neutral pH contributes the least towards enzyme activity as observed by the mean values, 0.352029, 0.192761 and 0.261289 at pH 5, 7 and 9. Similarly, increasing substrate concentration decreases the specific activity. A comparison of the mean values, 0.301341, 0.309857 and 0.194881 at 0.25%, 0.5% and 1% substrate concentration indicated the increase beyond 0.5% concentration significantly decreased specific enzyme activity. It may occur due to substrate inhibition that occurs when substrate is in excess. Finally, temperature has the highest effect on specific activity of the enzyme. An analysis of the mean values 0.557219, 0.126246 and 0.122614 at 30, 35 and 40 °C respectively, showed that increase in temperature decreased the specific activity, while 30 °C was considered to be the optimum temperature for enzyme activity. Figure 3 shows the variation on L-asparaginase activity at chosen levels.

Table 4. The effect of each factor on enzyme production expressed as mean specific activity calculated using SAS JMP Version 10.

Factor	Mean value		
	Level 1 IU/µg	Level 2 IU/µg	Level 3 IU/µg
Temperature	0.557219	0.126246	0.122614
Substrate	0.301341	0.309857	0.194881
pH	0.352029	0.192761	0.261289
Inoculum size	0.277981	0.259405	N/A

Analysis of variance (ANOVA) was used to determine how much variation is caused by individual factors (Table 5). It was found that temperature causes the most variation (85%) on L-asparaginase production followed by pH and substrate concentration, 8.7% and 5.6% respectively, while inoculum size produces the least variation (0.17%).

Partial purification of L-asparaginase enzyme

Ammonium sulfate precipitation was done with increasing saturation. It was found that the specific enzyme activity was highest at 60% saturation. Gel filtration was performed and different fractions were collected and analyzed, figure 4 indicates specific activity in each fraction. Fraction 11 showed highest specific activity of 1.89 IU/µg with an estimated molecular weight of 41.88 kDa with specific activity 1.586 U/µg, recovery rate 32% and 1.79-fold purity.

Figure 5 shows the standard curve used to calculate the molecular weight. Standard curve was made using anti-diuretic hormone, Bovine serum albumin, Myoglobulin, lysozyme, TyrGlyGly tripeptide, Azide and Lactoglobulin B proteins. Table 4 shows the purification profile of L-asparaginase produced from MH01.

Table 5. Analysis of Variance of different factors for optimization of L-asparaginase Production calculated using SAS JMP version 10.

Source	Nparm	DF	Sum of Squares	Variance	F Ratio	Prob > F	Percentage (%)
Inoculum Size	1	1	0.00155287	0.00155287	0.1121	0.7446	0.177140869
pH	2	2	0.07659303	0.0382965	2.7654	0.1107	8.737212963
Substrate Concentration	2	2	0.04925157	0.0246257	1.7782	0.2184	5.618284795
Temperature	2	2	0.74926641	0.3746332	27.0521	<.0001*	85.47122617

Characterization of Purified L-asparaginase

Effect of temperature and pH

Enzyme activity was measured at different ranges of incubation temperature and pH with the result plotted on graphs in figure 7 displaying error intervals around the mean at 95% confidence level ($p<0.05$) It was found that the optimum temperature of L-asparaginase, isolated from *Bacillus* MH01 strain is 30 °C with 95.76 IU/mL mean enzyme activity while retaining significant activity at physiological

temperature of 37 ºC i.e. ≥ 92 IU/mL (≥ 96% of mean). Similarly, the enzyme showed optimum activity at the pH 8.5 i.e. 81 IU/mL while retaining significant activity at physiological pH i.e. 70 IU/mL (≥86%).

Effect of substrate concentration and Kinetics of purified L-asparaginase

The effect of different substrate concentration was determined to construct a Lineweaver burk plot using linear regression on the reciprocal of substrate concentration and enzyme activity. The graph in figure 6, a Lineweaver burk plot with R value of 0.998 was used to calculate V*max* and K*m* value of the enzyme. It was found the enzyme had a K*m* value of 0.389 and V*max* value of 30395 µM/L.min with a *Kcat* value of 429 /s. **A** low K*m* of 0.389 mM means that the enzyme has a good affinity for its substrate, whereas a higher V*max* and K*cat* value of 30395 µM/L.min and 429/s, respectively indicated that the enzyme is significantly efficient.

Glutaminase activity

The presence of glutaminase activity was checked with 0.1 mL of 189 mM of glutamine as substrate with 1.8 µg of the enzyme. Glutaminase activity was found to be 6.34 IU/mL and the specific activity was 0.35 IU/µg which is moderately significant.

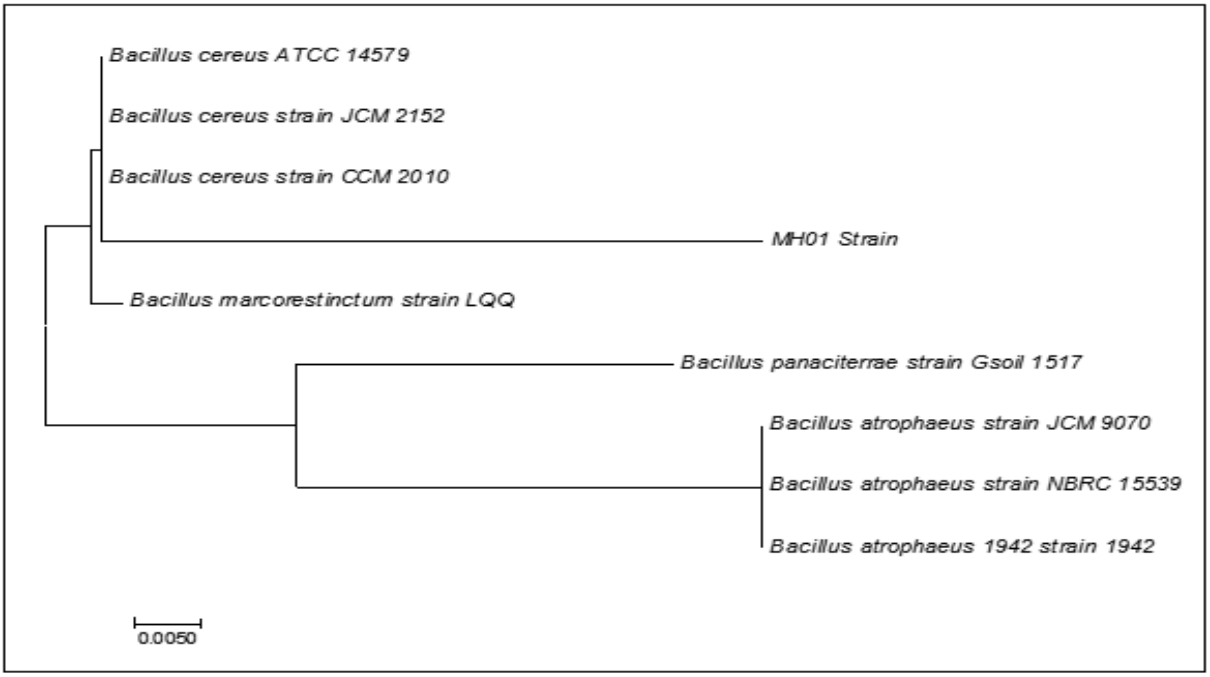

Fig. 1. Phylogenetic tree constructed using maximum likelihood method in MEGA version 7 showing MH01 strain as a species of *Bacillus*.

Discussions

The enzyme L-asparaginase has been subjected to decades of research dating back from Kid's experiments in 1953 with guinea pig serum and Broom's discovery of L-asparaginase linked with tumor regression in 1963 to several L-asparaginase preparations available in market for treatment of cancer. The medical application of L-asparaginase along with the discovery of its other application in reduction of acrylamide in foods has proved the great potential of this enzyme which in turn has raised its demand in recent years [2]. While L-asparaginase is isolated from a wide variety of sources ranging from plants to bacteria and fungi [23], only bacterial L-asparaginase is used in chemotherapy, whereas the potential of enzymes from other sources is yet to be researched or properly documented [2]. Similarly, research on various other strategies for the reduction of complications associated with therapy, such as acylation, mutagenesis to remove immunogenic epitopes and safe delivery mechanisms, is also inconclusive.

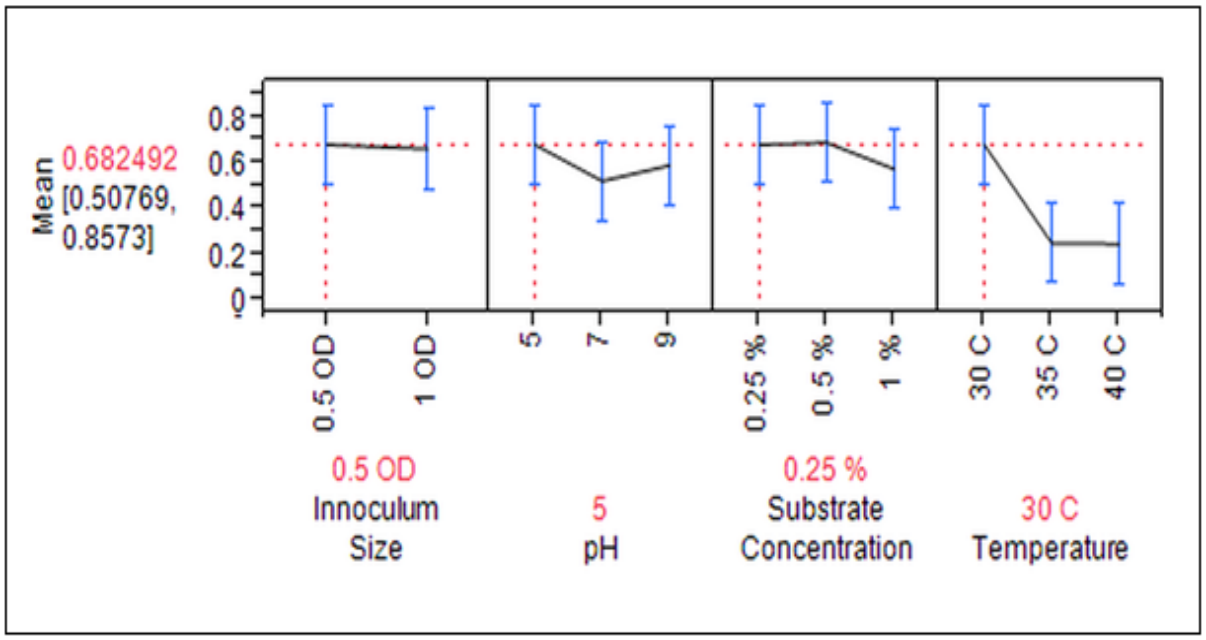

Fig. 2. Impact of selected factors on L-asparaginase production. Different levels in each factors are shown on x axis, whereas y axis shows specific enzyme activity. Graph produced by SAS JMP version 10.

The primary aim of this study was to demonstrate screening of environmental samples for L-asparaginase producing microorganisms followed by optimization of enzyme production by Taguchi DOE as an effective model for finding alternate L-asparaginase producing microorganisms. This model can be used to potentially find an ideal source of L-asparaginase which has the potential to replace the current commercially available options by exhibiting simplistic growth requirements while producing mass amounts of efficacious enzyme for use in either of its application. Soil samples from potato fields of Kasur, Pakistan were analyzed for L-asparaginase producing microorganisms by using Czapek Dox's media with bromothymol blue indicator. Since L-asparaginase breaks down L-asparagine in to L-aspartic acid and ammonia the latter raises the pH of the medium which is detected by the bromothymol blue indictor.

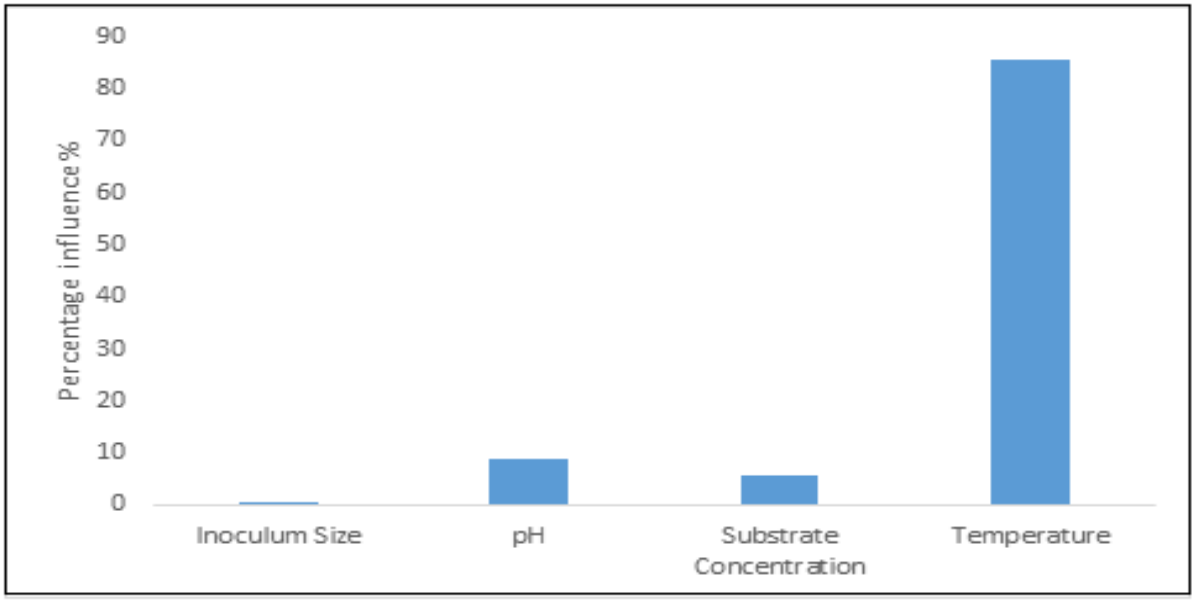

Fig. 3. Relative influence of each factor and interactions expressed as percentage on the production of L-asparaginase.

Different L-asparaginase producing microorganisms were isolated and their enzyme producing capability was compared by measuring specific enzyme activity at similar conditions. L-asparaginase activity is defined as amount of enzyme required to release 1 µmole ammonia produced per minute at 37 °C and was measured by quantifying ammonia by nesslerization [13]. Similarly, protein concentration was measured using Lowry method [14]. The isolate with highest enzyme producing capability was named as "MH01" strain. Furthermore, MH01 strain was subjected to various biochemical and molecular analysis. Using 16 S rRNA Sequencing. It was found that MH01 belongs to the genus *Bacillus*.

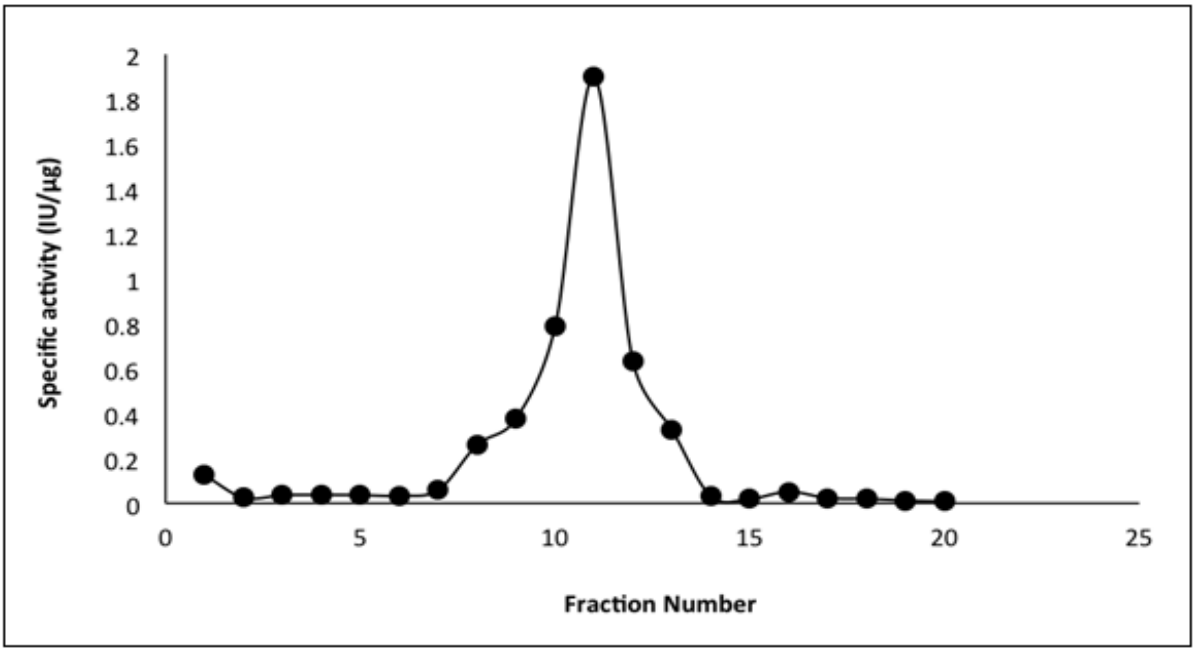

Fig. 4. Specific activity in each fraction of L-asparaginase isolated from *Bacillus* MH01 strain in size exclusion gel chromatography using Sephadex G-100 (10/50 mm).

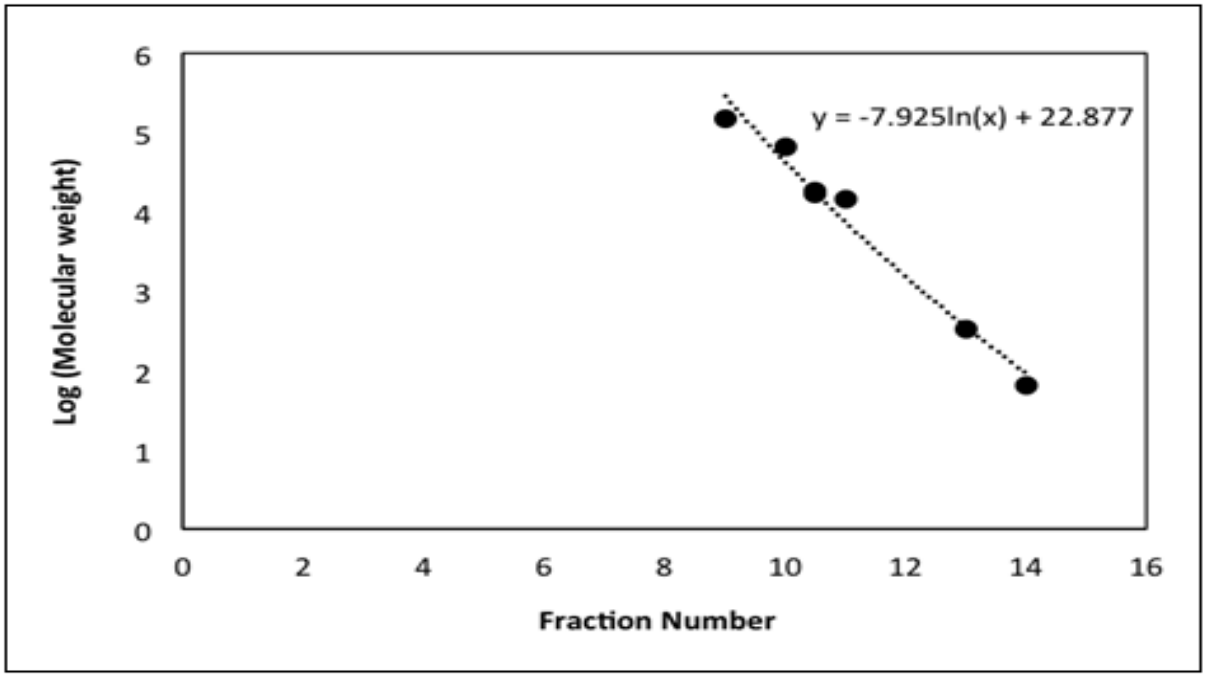

Fig. 5. Standard curve to estimate protein size obtained by eluting different sized proteins in column using Sephadex G-100 and taking Log molecular weight on y axis and fraction number with eluted protein on x axis.

The next step was to optimize the production of the enzyme. Since optimizing by one factor at a time is a tedious process, Taguchi design of experiments was chosen which has the advantage of deriving statistically valid conclusions in lesser number of experimental settings [19]. It also has the advantage of finding the effect of combination of different factors along with the individual contribution of each factor towards enzyme production. The enzyme was subsequently partially purified by ammonium sulfate precipitation and size exclusion gel filtration chromatography using Sephadex G-100.

The molecular weight was also determined by gel filtration chromatography by running different sized proteins to construct a standard curve.

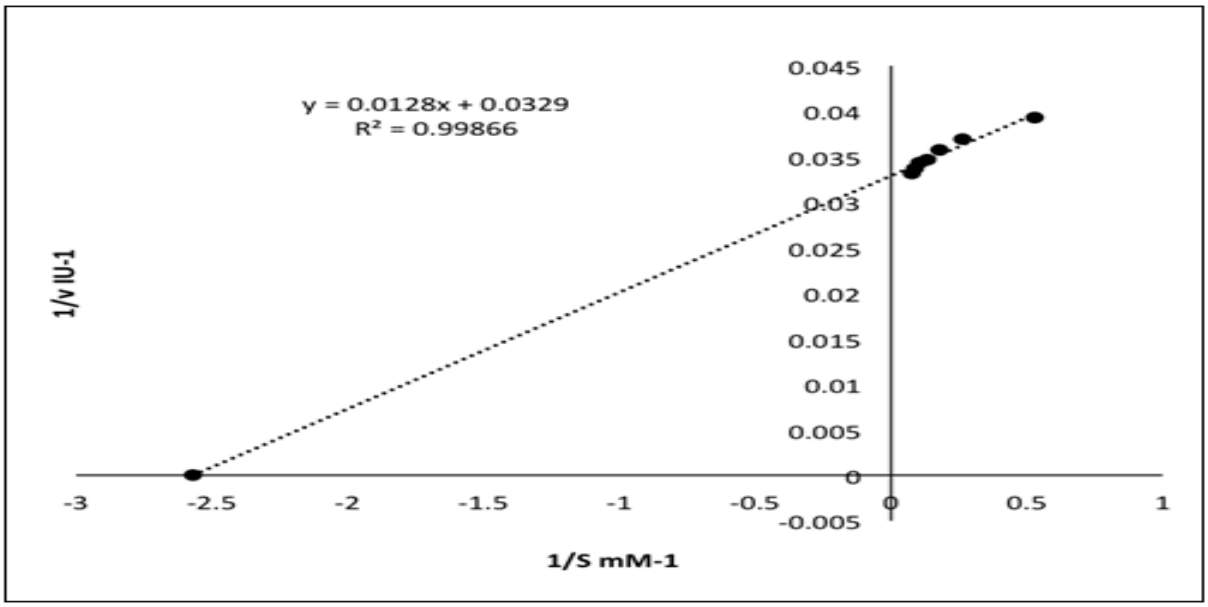

Fig. 6. Lineweaver Burk Plot of L-asparaginase isolated from *Bacillus* sp MH01 strain showing the enzyme has 0.389 mM K_m and 30395 μML^{-1} min^{-1} V_{max}.

According to Chan *et al.* [5] glutaminase activity may be required for some types of cancer, contradicting the previous belief that glutaminase activity is completely undesirable. Their study showed the glutaminase activity is required for cancer cells with functional L-asparagine synthetase gene. Thus, keeping this in mind L-asparaginase isolated from MH01 was tested for glutaminase activity and was found to be exhibiting moderate activity levels. In many aspects, *Bacillus* strain MH01 isolated using the prescribed model to find alternate L-asparaginase has proven itself to be a potentially right step in the direction towards the search for the ideal L-asparaginase producing strain by exhibiting generous levels of enzyme (71 IU/mL) with low levels of substrate (0.25%) and reaching a maximum specific activity of 1.5 IU/µg with the purification steps performed in this study as compared to the 0.2 IU/µg of commercially available *E. coli* L-asparaginase [24].

Similarly, the enzyme isolated in this study was potentially found to be comparable with a few commercially available options, such as L-asparaginase from *E. coli,* which is a homotetramer of 140kDa with 0.015 mM K*m* and 24 s^{-1} K*cat* [25-27], and Erwinase® which is L-asparaginase isolated from *Erwinia* is also a homotetramer of 140 kDa with a K*m* value of 0.096 mM and K*cat* value of 286.2s^{-1} [24, 28, 29]. L-asparaginase isolated from *Bacillus* sp. strain MH01 strain is 41.88 kDa in size with 0.389 mM K*m* and 429.3 s^{-1} K*cat* making it smaller in size and less likely to induce immunogenic response.

Though it has lower affinity as compared to *E. coli* and Erwinase®, it has a much greater turnover number which makes it an excellent candidate for further application studies. A lower K_m value means it has a high affinity for its substrate and a larger V_{max} and K_{cat} means it has a higher conversion rate.

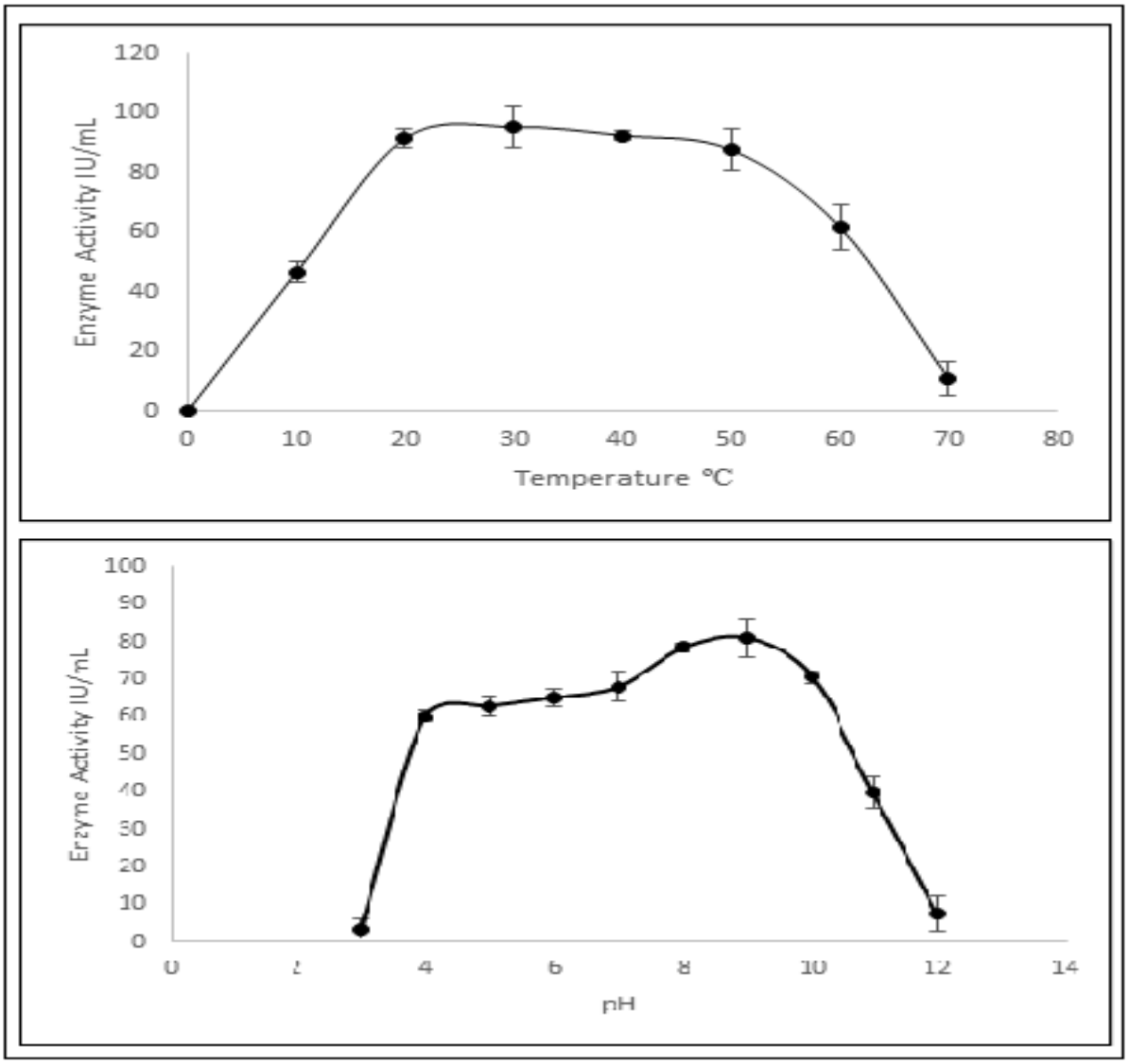

Fig. 7. Effect of temperature and pH on activity of L-asparaginase enzyme from *Bacillus* sp. strain MH01.

Conclusions

In conclusion, it can be said that the prescribed model can be readily used to find alternate L-asparaginase producing microorganisms, such as *Bacillus* MH01 strain which produces copious amounts of enzyme with relatively simplistic growth conditions and requirements. Similarly, enzymes from more strains can be isolated, purified and be subjected to clinical and industrial trials to fulfil the ultimate goal of producing an economical, efficacious, marketable enzyme which can either enhance the chemotherapy by L-asparaginase or effectively reduce acrylamide production in baking of starch containing foods.

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Conflicts of interest

The authors declare no conflicts of interest.

References

Narta UK, Kanwar SS, Azmi W. 2007. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia, Crit. Rev. Oncol. Hematol ogy **61(3),** 208-21.

Arif HM, Hussain Z. 2014. Important sources and medicinal applications of L-asparaginase, International Journal of Pharmaceutics Science Reviews **3,** 35-45.

Nagarethinam S, Nagappa AN, Udupa N, Rao V. 2012. Microbial L-Asparaginase and its future prospects, Asian Journal of Medical Research **1(4),** 159-168.

Vrooman LM, Supko JG, Neuberg DS, Asselin BL, Athale UH, Clavell L, Kelly KM, Laverdière C, Michon B, Schorin M, Cohen HJ, Sallan SE, Silverman LB. 2010. Erwinia asparaginase after allergy to E. coli asparaginase in children with acute lymphoblastic leukemia, Pediatric. blood. & cancer **54(2),** 199-205.

Chan WK, Lorenzi PL, Anishkin A, Purwaha P, Rogers DM, Sukharev S, Rempe SB, Weinstein JN. 2014. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells, Blood **123(23),** 3596-3606.

Kwon YM, Chung HS, Moon C, Yockman J, Park YJ, Gitlin SD, David AE, Yang VCL. 2009, Asparaginase encapsulated intact erythrocytes for treatment of acute lymphoblastic leukemia (ALL), Journal of Controlled Release **139(3),** 182-189.

Domenech C, Thomas X, Chabaud S, Baruchel A, Gueyffier F, Mazingue F. 2011. L-asparaginase loaded red blood cells in refractory or relapsing acute lymphoblastic leukaemia in children and adults: results of the GRASPALL 2005-01 randomized trial, British Journal of Haematology **153(1),** 58-65.

Gökmen V, Acrylamide Formation in Foods. 2016. Role of Composition and Processing, in Emerging and Traditional Technologies for Safe, Healthy and Quality Food, Springer, p 67-80.

Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA. 2002. Food chemistry: acrylamide from Maillard reaction products, Nature **419(6906),** 449-450.

Yaylayan VA, Stadler RH. 2005. Acrylamide formation in food: a mechanistic perspective Journal of AOAC International **88(1),** 262-267.

Anese M, Quarta B, Peloux L, Calligaris S. 2011. Effect of formulation on the capacity of l-asparaginase to minimize acrylamide formation in short dough biscuits, Food Research International **44(9),** 2837-2842.

Mahajan RV, Saran S, Saxena RK, Srivastava AK. 2013. A rapid, efficient and sensitive plate assay for detection and screening of l-asparaginase-producing microorganisms, FEMS. Microbiol letters, **341(2),** 122-126.

Imada A, Igarasi S, Nakahama K, Isono M. 1973. Asparaginase and glutaminase activities of micro-organisms Microbiology **76(1),** 85-99.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry **193(1),** 265-75.

Holt JG. 1977. The shorter Bergey's manual of determinative bacteriology. The shorter Bergey's manual of determinative bacteriology, 8th edition.

Kumar S, Stecher G, Tamura K, MEGA7. 2016. Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular. biol. evolution **33(7),** 1870-1874.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, Molecular Biology and Evolution **10(3),** 512-526.

Dehnad K., Quality control, robust design, and the Taguchi method. 2012. Springer Science & Business Media.

Nandal P, Ravella SR, Kuhad RC. 2013. Laccase production by Coriolopsis caperata RCK2011: optimization under solid state fermentation by Taguchi DOE methodology. Scientific reports, 3.

Roy RK. Design of experiments using the Taguchi approach: 16 steps to product and process improvement. 2001: John Wiley & Sons.

Andrew P. 2006. Estimation of molecular size and molecular weights of biological compounds by gel filtration. Methods of Biochemical Analysis, Volume **18,** p 1-53.

Andrews P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration, Biochemical Journal **91(2),** 222.

Batool T, Makky EA, Jalal M, Yusoff MM. 2016, A comprehensive review on L-asparaginase and its applications, Applied biochemistry and biotechnology **178(5),** 900-923.

Zuo S, Xue D, Zhang T, Jiang B, Mu W. 2014. Biochemical characterization of an extremely thermostable l-asparaginase from Thermococcus gammatolerans EJ3, J. Molecular Catalysis B: Enzymatic 122-129.

Derst C, Henseling J, Röhm KH. 2000. Engineering the substrate specificity of Escherichia coli asparaginase II. Selective reduction of glutaminase activity by amino acid replacements at position 248, Protein Science **9(10),** 2009-2017.

Sanches M, Sanches M, Barbosa JA, de Oliveira RT, Abrahão Neto J, Polikarpov I. 2003. Structural comparison of Escherichia coli L-asparaginase in two monoclinic space groups, Acta Crystallographica Section D: Biological Crystallography **59(3),** 416-422.

Wriston Jr JC, Yellin TL. 1973. L-asparaginase: a review. Adv Enzymol Relat Areas Journal of Molecular Biology **39,** 185-248.

Belur PRKPD. 2010. Partial purification and characterization of. Pediatr, Blood Cancer **54,** 199-205.

Phillips RM, Saleem MU, Williams A, Morgan J. 2013. Kinetic analysis of the glutaminase activity of L-asparaginases derived from *Erwinia chrysanthemi* (Erwinase) and *Eshericia coli* (Kidrolase), AACR Annual Meeting., (Abstract C154.).