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Isolation and Anti-Leukemic Characterization of Extracellular L-asparaginase From Endophytic Bacterium, *Brevibacterium* sp. M-R21 Isolated *Glycyrrhiza glabra* Root

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Abstract: L-Asparaginase (L-ASPase) is known as a potent anti-cancer drug against L-Asparagine-auxotroph tumor cells. In this study, an endophytic L-ASPase producing bacterium of the genus *Bervibacillus* from the root of *Glycyrrhiza glabra* was screened and characterized. After purification of the enzyme by ammonium sulfate precipitation, dialysis, and silica gel column chromatography, anti-cancer studies were performed against MRC-5 (normal lung cells) and U937 cell (leukemia cell line). Additionally, optimization fermentation was performed in terms of significant variables screened from a one-factor-at-the-time (OFAT) approach. The interactions of different experimental parameters were investigated using the response surface methodology (RSM) with the central composite design (CCD) algorithm. Cytotoxicity study showed that the dose-dependent effect of the L-ASPase at 100 IU/ml had a lethality of about 80% against leukemia cells. Therefore, the IC_{50} of the enzyme for leukemia cells was calculated to be approximately 33.54 IU/ml. Interestingly, the cytotoxicity of L-ASPase against normal lung cells was only about 20% at L-ASPase activity of 60-100 IU/ml. Based on the quadratic model, the optimal fermentation conditions were predicted to be 2% glucose, 2% NaCl, pH7, and incubation temperature 30 °C. Under these conditions, the highest enzyme activity was 90 IU/ml, which had an efficiency of about 30% compared to non-optimized conditions. The results showed that L-ASPase isolated from *Brevibacterium* sp. M-R21 with selective cytotoxicity against the leukemia cell line may be a potential candidate as an anti-cancer drug after further study.

Keywords: L-Asparaginase; Anti-Leukemia activity; *Brevibacterium* sp. M-R21; Response Surface Methodology (RSM).

1. Introduction

L-ASPase is one of the approved drugs for the treatment of soft tissue cancers such as lymphomas and leukemias. In addition, it has been shown that this enzyme can effectively stop the growth of some tumors, especially liver carcinoma (HepG2) and colon cancer (Hct-116) [1, 2]. In fact, L-ASPase belongs to the group of amidohydrolase enzymes (E.C.3.5.1.1), catalyzing L-Asn to aspartic acid and ammonia. L-ASPase is found in all living organisms,

including animals, plants, and microorganisms. In general, two types of L-ASPase have been identified in living organisms, including cytoplasmic L-ASPase or type I and secretory ones or type II. L-ASPase type I can catalyze L-glutamine in addition to L-Asn, while extracellular L-ASPase or type II intends a high affinity for L-Asn other than L-glutamine with a more specific catalytic activity [3, 4].

Some cancer cells have lost the ability of L-Asn biosynthesis due to mutations in the L-ASPase synthase gene. Therefore, L-Asn present in the bloodstream is required to proliferate and expand the tumor. The pharmacodynamic of L-ASPase is such that the enzyme triggers tumor death by depriving tumor cells of access towards extracellular asparagine [5-7]. Currently, two bacterial L-ASpases obtained from *E. coli* and *Erwinia* are used in combination with chemotherapy to treat acute lymphocytic leukemia (ALL) and lymphosarcoma [8, 9]. However, commercially available L-ASpases have impediments due to their cross interactions, immune system stimulation, drug resistance, and nonspecific L-glutaminase activity [10]. Therefore, side effects associated with L-ASPase can lead to widespread liver dysfunction, pancreatitis, leukopenia, diabetes, neurological seizures, and coagulation abnormalities, and intracranial thrombosis [11]. Therefore, it seems reasonable why researchers are trying to find new L-ASPase with more desirable properties and minimal side effects [12]. Various reports have shown that bacterial-derived L-ASPases have more effective anti-leukemic properties and fewer side effects than other isoenzymes [13]. Endophytes are microorganisms that coexist with plants, and, to date, various metabolites have been isolated from them [14]. In this study, licorice endophytes were isolated from the plant root, and their ability to produce the L-ASPase was investigated. Licorice is one of the well-known plants that is widely used in traditional medicine. The medicinal properties of licorice are attributed to its root extract [15, 16]. Besides, the licorice root is enriched with amino acids such that it could be a rational option for inhabiting endophytes that consume L-Asn as a source of carbon and nitrogen [17, 18]. With this assumption, licorice was selected to maximize the chance of isolating L-ASPase producing endophytes, because L-ASPase-positive bacteria are far more likely to be found in places where the amino acid asparagine is abundant.

2. Materials and Methods

2.1. Media and chemicals.

Phenol red was purchased from Sigma; agar was purchased from Merck; other materials were of laboratory grade as obtained. The M9 basal salt medium used for L-ASPnase production included the following (for 100 ml): Glucose 1 g; L-Asn 0.1 g; K_2HPO_4, 0.05 g; 0.001 g, $FeSO_4$; $CaCl_2$. $2H_2O$. $7H_2O$, 0.001 g. For preparing M9 agar, 1.5 g of agar was added to M9 basal ingredients.

2.2. Plant collection.

The medicinal plant, *Glycyrrhiza glabra* (licorice), was collected Central District, Khorramabad, Lorestan Province, Iran (33°28'24.2"N 48°20'37.5"E). The wet root of the plant was collected in the plastic bags and transferred to the laboratory.

2.3. Bacterial isolation.

The endophytic bacteria were isolated from the *Glycyrrhiza glabra* root, as described previously [19]. Briefly, the roots were sterilized by 75% (v/v) ethanol for 5 min and sodium hypochlorite 0.1% for 30 sec. After that, the roots were ground in the mortar, suspended on the sterile water. The suspension was serially diluted to $10^{-3,}$ and 100 µl were spread on the Muller Hinton agar (MHA) plates. The plates were incubated at 37 °C for 48 h.

2.4. Screening of asparaginase producing bacteria.

L-ASPnase producing colonies were identified by colony spotting method on the surface of the M9 agar medium supplemented with 0.01 g/l phenol red as a pH indicator. After 48 h incubation, the L-ASPnase-positive colonies were isolated based on creating a pink zone due to ammonia production in the agar medium with a yellow background. To confirm L-ASPnase activity, the isolate was cultured in M9 broth medium. After 48 h, the culture medium was turned from yellow to pink color [4].

2.5. Identification of asparaginase producing bacterium.

Primary identification was performed using biochemical studies. In addition, 16S rDNA gene sequencing was conducted to determine phylogenetic relationships. For this purpose, the first genomic DNA was extracted by DNA extraction kit (Qiagen, Germany). Then, 16S rDNA gene was amplified by PCR with one pair of primers, including forwarding (5-AGAGTTTGATCCTGGCTCAG-3) and Revers (5-AGGAGGTGATCCAGCC-3). The PCR reaction kit contained a master mix and loading dye from Wizol Company (South Korea). DNA amplification was performed as follows: initial denaturation at 94 ºC for 5 min followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 45 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 5 min [20]. After that, the PCR product was electrophorized on 1% agarose and stained by safe stain (Sinaclon Co., Iran). The purified PCR product of the 16S rRNA gene was sequenced based on the Sanger method by Sinagene Company (Tehran, Iran). Nucleotide sequences were reviewed and edited by BioEdit software version 7.0.5 and BLASTed by blastn software in the NCBI Genbank website (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). After identifying the bacterium, its 16S rDNA fragment sequence was submitted to NCBI for assigning an accession number.

2.6. L-ASPnase assay in broth medium.

L-ASPnase activity was studied by the Nessler method in the bacterial culture medium. For this, the bacterium was cultured in a 250 ml flask containing 100 ml of M9 liquid medium and incubated in a shaker at 100 rpm at 37 °C for 48 h. The bacterial cells were then centrifuged at 25,200 ×g for 15 min, and the supernatant was taken to evaluate L-ASPnase activity. Briefly, 1 ml of supernatant was added to 2.0 ml of sodium borate buffer (0.1 M, pH 8.5) in a glass tube. Then, 1 ml of L-Asn solution (0.05 M) was added to the tube. The reaction tube was incubated at 15 °C for 15 min, and then the reaction was stopped with 0.5 ml of trichloroacetic acid (TCA, 15%). The reaction tube was centrifuged at 11,200 ×g for 15 min to precipitate the inactivated enzyme. Finally, the solution absorbance was measured at 450 nm, and the L-ASPnase activity was calculated using the equation obtained from the standard curve [21].

2.7. Enzyme extraction and purification.

The 48-h cultured broth medium was centrifuged at 8000 g for 15 minutes at 4 °C to precipitate bacterial cells. The supernatant containing crude enzyme was taken for subsequent purification steps. A volume of 100 ml of CFS was mixed with an equal volume of 70% ammonium sulfate solution and kept in an ice bath for 30 minutes. After that, the mixture was centrifuged at 4500 g for 15 minutes. The precipitate was then dissolved in phosphate buffer (PBS) (0.1 M, pH~7) and dialyzed against the increased volume of PBS (0.01 M). The dialysate was subjected to a Sephadex G-100 column (920×1.25 cm). Briefly, 10 ml of dialysate was loaded over the column and eluted with 0.1 M Tris-HCl buffer (pH 8) so that 20 fractions were collected per 10 min. Subsequently, the resulting fractions were examined for L-ASPnase activity and protein assays. The amount of total protein at each stage of purification was measured using the Bradford method from a standard curve constructed by bovine serum albumin (BSA) [21, 22]. The enzyme unit (U) for L-ASPnase expresses the amount of enzyme which releases 1 mol of ammonia per min. The specific activity is defined as the number of enzyme units per milligram of protein.

2.8. Antileukemia activity assay.

The anti-tumor activity of the purified L-ASPnase enzyme was investigated on MRC-5 (normal lung cells) and U937 cell (leukemia cell line) [10]. The IC_{50} was determined by treating different concentrations of the enzyme on the cell lines and measuring their survival by MTT method [10]. Briefly, a number of 10^4 cells with 80% confluency were seeded in 96-well-plate containing 150 µl RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). A volume of 50 µl containing different concentrations of L-ASPnase was added to each well. The plate was incubated at 37 °C in a CO_2 incubator for 48 h. After that, 10 µl of MTT reagent was added in each well, and the plates were incubated again for 4 h under the same conditions. The produced formazan crystals were precipitated by centrifugation at 250 ×g. After removing the supernatant, the formazan blue crystals were dissolved with 150 µl of DMSO. The absorbance of the formazan solution was measured at 570 nm by a microplate reader [21]. The viability of treated cells was calculated by the following equation:

$$\text{Viability}(\%) = \frac{\text{Treated sample absorbance}}{\text{Control sample absorbance}} \times 100$$

2.9. Optimization of L-ASPnase production.

2.9.1. Preliminary screening of effective factors.

Factors affecting the L-ASPnase production by the bacterium were determined based on one-factor-at-the-time (OFAT) under submerged fermentation. Preliminary studies without considering the interaction of various factors such as incubation time (12, 24, 48 and 72 h) temperature (20, 25, 30, 35 and 40 °C), pH (5, 6, 7, 8 and 9) and salinity (0, 1, 3 and 5%) as well as nitrogen (0.1, 0.25, 0.5 and 1 mg/l) and carbon (glucose) (0.5, 1, and 3 %) sources on L-ASPnase activity were investigated. All experiments were performed in triplicate.

2.9.2. Response surface method optimization.

The variables with the greatest impact on L-ASPnase activity extracted from the primary optimization method were selected for experimental design. These variables included initial pH (4–10), temperature (25–35 °C), NaCl (1–3 %) and glucose (1-3 %). Statistical optimization was designed based on response surface methodology with the central compound design algorithm (CCD). Based on the experimental pattern, 30 total runs included 6 center points, 8 axial points, and 24 factorial points (Table 3). The axial points were considered as rotatable points, which confirm any curvature in the response. Analysis of variances (annotated ANOVA) was conducted to analyze the statistical significance of the model, and each term [21, 22]. A second-order polynomial model was predicted from RSM demonstrating linear, quadratic, and interaction effects of variables on the response (L-Asp activity) as follows:

$$Y = \beta_0 + \sum_{i} \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

Where Y is the predicted response (L-ASPnase activity) around the variable levels of Xi and Xj, other terms including β0, βi, βii, and βij represent the constant coefficients of intercept, linear, squared, and interaction effects, respectively. β_0 is the constant coefficients of intercept; β_i, β_{ii} and β_{ij} represent coefficients of the linear, quadratic and interactive terms, respectively.

3. Results and Discussion

3.1. Isolation and identification of L-ASPnase producing bacterium.

Forty-five different endophytic strains were examined for L-ASPnase production using a modified M9 broth medium (Figure 1). Here, an isolate with the highest L-ASPnase activity was selected for further studies.

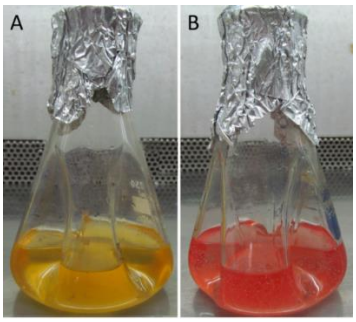

Figure 1. M9 broth medium. A) Before the cultivation of L-ASpase producing bacterium and B) After production of L-ASpase by the bacterium in 24-h incubation.

3.2. Bacterium characterization.

According to genetic alignment, approximately 99% of the sequence similarity between the isolated bacterium and other *Brevibacterium* species was established. Figure 2 shows the isolated phylogenetic relationship with different species of *Brevibacterium*. As can be seen, according to the similarities percent of the bacterium with the *Brevibacterium* genus, the associated 16S rRNA gene was deposited in the NCBI GeneBank called *Brevibacterium* M-R21 with the accession number MT749247.

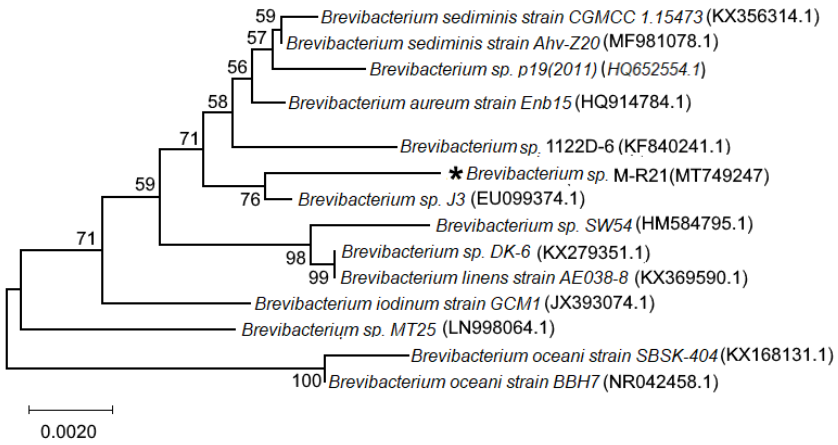

Figure 2. Phylogenetic tree displays neighboring relations between *Brevibacterium* M-R21with the closest 16S rRNA gene sequences retrieved from NCBI. The isolated bacterium was labeled as a black star. The bootstrapping value was generated from 1000 replicates, which are presented as branch distance from the clades.

Brevibacterium is a gram-positive bacterium inhabiting soil belonging to the Actinomycetales family. The closest genus to *Brevibacterium* is Corynebacterium, from which various metabolites are obtained. *Brevibacterium flavum*, for example, now classified as Corynebacterium glutamicum, is one of the most applicable organisms for producing glutamic acid and lysine [23]. Although several metabolites, particularly various enzymes, have been isolated from other actinomycetes, this study is the first report of L-ASPnase activity of a bacterium of the *Brevibacterium* genus.

3.3. L-ASPnase extraction and purification.

The extracellular enzyme secreted by the bacterium in the M9 broth medium was purified by steps including ammonium sulfate precipitation, dialysis, and gel filtration column chromatography onto Sephadex G-100 (Sigma, USA). The crude enzyme indicated a specific activity of 0.14 IU/mg, while after precipitating by ammonium sulfate, enzyme activity reached 0.36 IU/mg. With the desalination of the resultant by dialysis, enzyme activity increased 0.52 IU/mg, and ultimately purified enzyme was obtained by column chromatography showing 0.67 IU/mg specific activity (Table 1). As seen in Table 1, the enzyme activity with increasing purity arose about 4.7 folds compared to the crude enzyme.

Table 1. Purification process of L-ASPase from M9 broth and yield of each step.

Step	Volume(ml)	Total protein(mg/ml)	Activity(IU/ml)	Specific activity(IU/mg)
Crude extract	100	0.53	0.051	0.14
Ammonium sulfate precipitation	56	0.44	0.44	0.36
Dialysis	6.5	0.32	0.32	0.52
Chromatography	1.5	0.17	0.18	0.67

3.4. Purification assay of L-Asp enzyme.

The purified enzyme was subjected to SDS-PAGE for determining its molecular weight. As seen in Figure 3, the molecular weight of the purified enzyme was estimated at 43 kDa. Based on the other studies, most L-ASPnase isolated from gram-positive bacteria, especially the *Bacillus* genus, had molecular weight within 35-47 kDa [24-27]. For instance, Roy *et al*. (2018) isolated an L-ASPnase enzyme from *B. megaterium* with a molecular weight

of 47 kDa on SDS-PAGE [28]. Sudhir *et al*. (2016) purified an L-ASPnase from *B. licheniformis* with 37 kDa showing a potent antineoplastic activity [24]. Zhang *et al*., 2015 cloned a new L-ASPnase from *B. megaterium* in *E. coli* that its molecular weight was 39.63 kDa [25]. Our study showed that L-ASPnase obtained from *Brevibacterium* is probably similar to those enzymes isolated from *Bacillus* species.

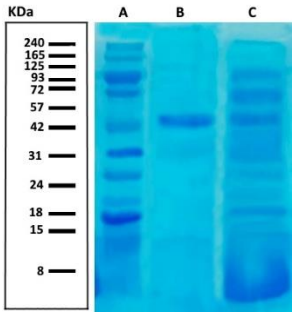

Figure 3. SDS-PAGE of purified L-ASPase. Lane A shows protein weight Marker, Lan B displays purified L-Asp enzyme from G-100 column, and Lane C- crude enzyme (unpurified).

3.5. Anti-cancer activity of L-ASPnase.

The cytotoxicity of L-ASPase isolated from microorganisms and plants against a wide range of cell lines has been confirmed in numerous studies [1, 13, 27]. In 2018, Moharib examined the effect of a plant-derived L-ASPase isoenzyme on leukemia and Hela cells. They concluded that the inhibitory effect of the enzyme on HepG2 was significantly greater than Hela cells [29]. Numerous studies have shown that L-ASPase derived from microbial sources, unlike plants, exert more toxicity effect on the cells [13]. One study reported the toxicity effect of L-ASPase isolated from *Aspergillus oryzaei* against Hela cells [30]. Another similar study showed that an L-ASPase from *Aspergillus flavus* inhibited only 50% of leukemia cells [31]. In this study, L-ASPase isolated from *Brevibacterium* could inhibit the growth of the leukemia cell line (U937) in dose-dependent mode (Figure 4). In addition, the toxicity effect of the L-ASPase was significantly increased against the normal cell line (MRC-5).

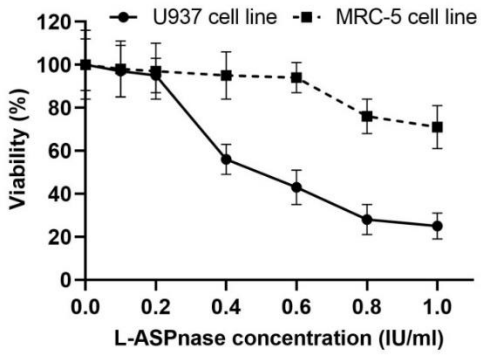

Figure 4. Cytoxicity of purified L-ASPase from *Brevibacterium* M-R21 against U937 and MRC-5 cell lines.

Therefore, our study showed that L-ASPase from *Brevibacterium* exerts a selective inhibition against the different cell lines. Studies have shown that high enzyme activity does not mean that the enzyme is suitable for pharmaceutical use. The stability of L-Asp activity under physiological conditions such as pH and osmolarity are the main criteria in selecting the enzyme as an anti-leukemia drug [3, 13, 32]. Therefore, the most significant analysis is to determine the anti-cancer activity of the enzyme in the tumor cells in vivo. However, the

findings of this analysis indicate that the enzyme produced by *Brevibacterium* sp. M-R21 has relatively appropriate properties, although more thorough and in-depth studies are required.

3.6. OFAT experiment.

OFAT optimization method was used to screen for significant variables on bacterial L-ASPase activity. Figure 5 shows the results of 6 different variables on L-ASPase activity. Of these parameters, four were selected with the most significant effect for statistical analysis of surface response. Therefore, the OFAT method is first used to investigate the effect of various factors on microorganisms. In this method, the interaction of factors is not considered, and only evaluates one case at a certain time without considering others [33]. In this study, the factors affecting L-ASPase activity included pH, temperature, NaCl, and glucose (as a carbon source). Examining the effect of carbon and nitrogen source, it was found that 3% glucose concentration could increase L-ASPase activity, while nitrogen (ammonium chloride) concentration had no effect on L-ASPase activity. Studies have shown that glucose in culture media increases enzymatic activity. Besides, to economically produce L-ASPase, glucose is preferred over other carbon sources [34].

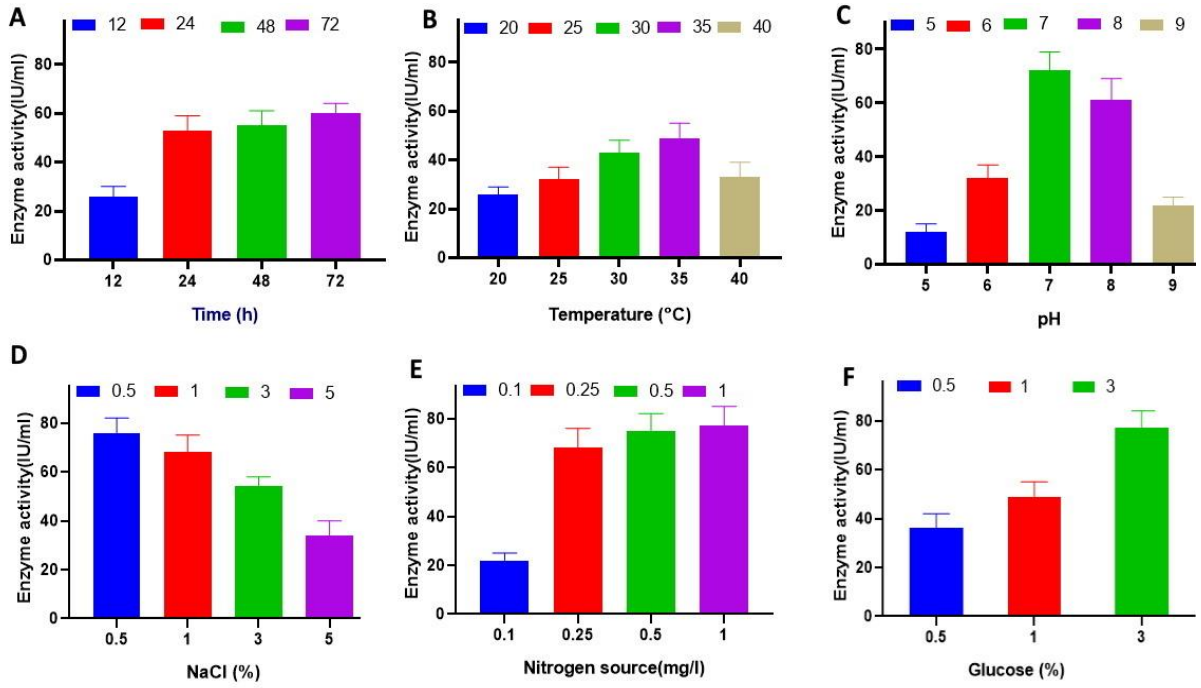

Figure 5. The results of OFAT experiments. Six factors affecting L-Asp activity were analyzed in the different levels, and the significant items were considered for the RSM study.

3.7. Optimization based on RSM (CCD).

In accordance with the surface response analysis method calculated on the CCD, four effective factors were investigated to optimize the production of L-ASPase by the bacterium. The results obtained from 30 experiments proposed by the CCD based on the following regression equation:

$$Y = -1096.68 + 32.14\ X_2 + 37.62\ X_3 + 16.50\ X_4 - 0.37\ X_1 X_2 - 0.27\ X_2 X_3 + 1.10\ X_2 X_4 - 11.68\ X_1^2 - 0.50\ X_2^2 - 8.68\ X_3^2 - 9.93\ X_4^2$$

The relationship between variables on L-ASPase activity was analyzed based on annotated ANOVA by DOE software to predict the effects of each variable alone and their

interactive mode. As seen in Table 2, the proposed quadratic model was significant that predicts the responses based on a second-order polynomial equation. Regression analysis around the polynomial model showed the significance of factors B, C, D, AB, BC, BD, A2, B2, C2, and D2 in the distance of 5% (P-value <0.05). The meaning of the interactive terms AB, BC, and BD means that the interactive effects of pH on temperature, the temperature on NaCl, and temperature on glucose may affect L-ASPase activity. In addition, the non-significance of lack of fit (LOF) (P> 0.05) means that the predicted results are consistent with the model [22]. The precision of predicting outcomes based on the present model is accomplished by measuring the difference between the adjusted R-square and the predicted R-square. Based on this, less variance implies a more accurate prediction and more consistent model-calculated performance [22, 34]. In comparison, the Adeq value of 46.139 shows the high accuracy of the results, which, according to the 4-precision model, describes the adequate accuracy of the measurement (Ad-P>4).

Table 2. Statistical analysis based on annotated ANOVA for L-ASPase activity by Brevibacterium sp. M-R21.

Source	**Terms**	***p*-value**	**Source**	**Terms**	**p-value**
Model	quadratic	< 0.0001*	BC	Interactive	0.0143
pH(A)	Linear	0.8398	BD	Interactive	< 0.0001*
Temperature(B)	Linear	< 0.0001*	CD	Interactive	0.3296
NaCl (C)	Linear	0.0006*	A2	Squared	< 0.0001*
Glucose (D)	Linear	< 0.0001*	B2	squared	< 0.0001*
AB	Interactive	0.0018	C2	Squared	< 0.0001*
AC	Interactive	0.4615	D2	squared	< 0.0001*
AD	Interactive	0.6217	LOF	-	0.0759

Parameter	**Value**		**Parameter**	**Value**
Std. Dev.	0.020		R-Squared	0.9958
Mean	0.34		Adj R-Squared	0.9918
C.V. %	5.88		Pred R-Squared	0.9777
PRESS	0.031		Adeq Precision	46.139

Table 3. Optimization of L-ASPase activity using RSM based on CCD by *Brevibacterium* sp. M-R21.

Run	**pH (A)**	**Temperature (B) (°C)**	**NaCl (C) (%)**	**Glucose (D) (%)**	**Predicted (IU/ml)**	**Actual (IU/ml)**
1	8.00	25.00	1.00	3.00	50.72	48.32
2	6.00	25.00	1.00	3.00	47.16	45.41
3	8.00	35.00	1.00	1.00	31.99	32.19
4	7.00	30.00	2.00	0.00	23.61	24.06*
5	6.00	35.00	1.00	1.00	37.21	35.12
6	6.00	25.00	3.00	1.00	32.21	31.42
7	7.00	20.00	2.00	2.00	30.40	33.11
8	8.00	35.00	3.00	1.00	25.53	27.15
9	7.00	30.00	2.00	2.00	88.26	88.08
10	8.00	25.00	3.00	1.00	35.96	34.38
11	8.00	25.00	3.00	3.00	51.58	53.54
12	7.00	30.00	2.00	2.00	88.26	87.04
13	6.00	35.00	3.00	1.00	29.41	31.81
14	6.00	25.00	1.00	1.00	34.49	34.21
15	7.00	30.00	4.00	2.00	50.53	48.43
16	6.00	35.00	1.00	3.00	71.82	73.40
17	8.00	25.00	1.00	1.00	36.90	36.38
18	7.00	40.00	2.00	2.00	44.64	42.06
19	7.00	30.00	2.00	2.00	88.26	88.12
20	5.00	30.00	2.00	2.00	42.00	42.31
21	8.00	35.00	1.00	3.00	67.75	68.40
22	7.00	30.00	2.00	4.00	73.83	73.51
23	9.00	30.00	2.00	2.00	41.68	41.5
24	7.00	30.00	0.00	2.00	57.47	59.71
25	6.00	35.00	3.00	3.00	65.82	66.21
26	8.00	35.00	3.00	3.00	63.09	63.37
27	7.00	30.00	2.00	2.00	88.26	87.05
28	6.00	25.00	3.00	3.00	46.67	46.47

Run	pH (A)	Temperature (B) (°C)	NaCl (C) (%)	Glucose (D) (%)	Predicted (IU/ml)	Actual (IU/ml)
29	7.00	30.00	2.00	2.00	88.26	90.11**
30	7.00	30.00	2.00	2.00	88.26	89.16

All responses associated with run orders have presented in Table 2. As can be seen, the lowest and highest responses were determined to run order 4 and 29 that were 24.06 and 90.11 IU/ml, respectively.

Three-dimensional plots (3D) represent the significant interaction of independent variable pairs (Figure 6). These plots provide a better understanding of the polynomial model in the interactive situation between independent factors. The highlights are determined based on the interactive powers of each of the independent variables [22].

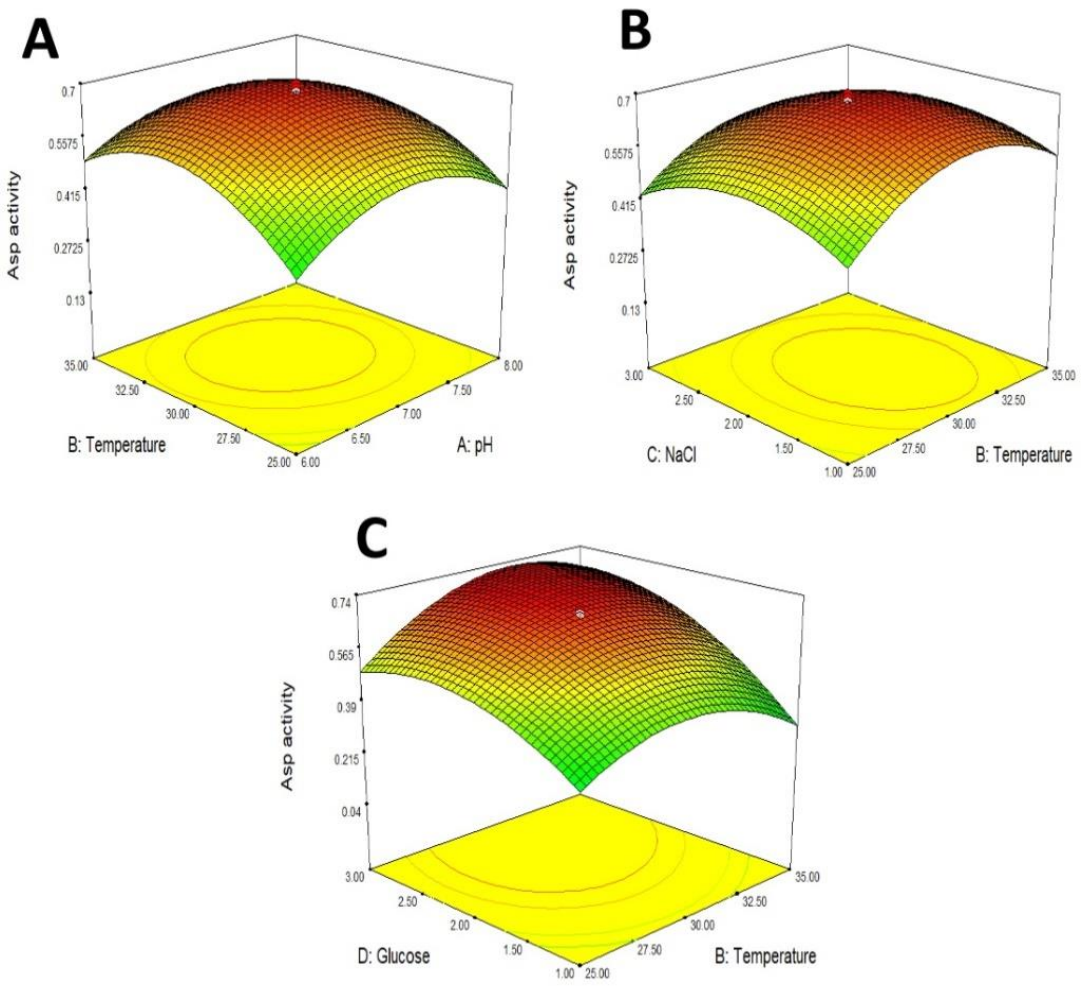

Figure 6. 3D plots show interactive effects between variables: A) temperature and pH with a polynomial curvature; (b) NaCl and temperature with polynomial (c) glucose and temperature with significant polynomial and curvature.

In the present study, the specific activity was obtained in optimal condition about 90 IU /mg, which was similar to commercial L-ASPase produced by *E. coli* reported with about 85 IU/mg. These increased activities were related to the central points, including pH 7, temperature 30 °C, NaCl 2%, and glucose 2%. The lowest activity of L-ASPase was obtained at pH 7, temperature, 30 °C, NaCl, 2%, lacking any carbon source, i.e., glucose. This was higher than the value reported by Dias *et al*. (2016), who obtained 67.49 U mL1 for L-ASPase activity from *Aspergillus oryzae* CCT 3940 after optimization of process conditions [34].

Optimization for the L-ASPase production by the OFAT method revealed that the incubation time and nitrogen source had no significant effects on bacterial L-ASPase activity. However, L-ASPase activity increased significantly with changes in pH. This trend was also confirmed in the statistical optimization method, where the highest L-ASPase activity occurred in neutral pH. Besides, the temperature affecting the L-ASPase production was 30 °C, although L-ASPase activity remained high in the temperature range of 30 to 40 °C. In our previous report, *Rouxiella* sp. AF1 isolated from farmland soils had the best L-ASPase activity at 30 °C, pH 7, NaCl 3%, and glucose 1% [21]. Some reports have claimed that many *Bacillus* genus members showed L-ASPase activity in the pH range of 6-8 and temperature 37 °C [26, 27, 35]. Significant reductions in L-ASPase activity at low pH may be attributed to change in enzyme affinity to the substrate [36].

4. Conclusions

Due to the relatively favorable anti-tumor activity of L-ASPase produced by *Brevibacterium* sp. M-R21 could potentially be used for pharmaceutical use as well as in the food industry to remove acrylamide. In addition, optimization experiments with a combination of OFAT and a central composite design provided a reliable approach for evaluating enzyme production. Although Extraction and purification of L-Asp yielded low enzyme by ammonium sulfate precipitation, dialysis, and then column chromatography, its purity showed to be significantly satisfactory. Taken together, this study showed that the use of endophytes isolated from medicinal plants could be a good alternative to find new metabolites, especially L-Asp, for pharmaceutical applications.

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Conflicts of Interest

The authors declare no conflict of interest.

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