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EXPERIMENTAL ARTICLES

Optimization of Culture Media for L-Asparaginase Production by Newly Isolated Bacteria, *Bacillus* sp. GH5[1]

Saeed Gholamian[a, 2], Soudabeh Gholamian[a], A. Nazemi[a], and M. Miri Nargesi[b]

[a] *Microbiology Department, Islamic Azad University Tonekabon Branch, Tonekabon, Iran*

[b] *Biomedical Science Department, Universiti Putra Malaysia, Selangor, Malaysia*

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Abstract—The enzyme L-asparaginase has been extensively studied by many researchers mainly because of its considerable therapeutic properties. Producing a convenient quantity of L-asparaginase can be conducted either by discovering new microbial sources with higher enzyme production or by manipulating the medium components for known microbial sources. The present paper discusses the studies carried out in order to enhance the production of L-asparaginase by newly isolated bacteria, *Bacillus* sp. GH5. Based on the results obtained from media optimization studies, a modified media was developed for optimal L-asparaginase production. Concisely, screening of the nutrients using a proper statistical design showed that tapioca starch, gelatin, ammonium oxalate, $CaCO_3$, and L-asparagine were respectively the most important sources for carbon, organic nitrogen, inorganic nitrogen, mineral salt, and amino acids. The composition of the optimized medium was the following (per 1 L): 5.0 g L-asparagine; 0.5 g $MgSO_4 \cdot 7H_2O$; 6.0 g $NaHPO_4 \cdot 2H_2O$; 3.0 g $(NH_4)_2C_2O_4$; 0.5 g $CaCO_3$; 0.014 g $CaCl_2 \cdot 2H_2O$; 2.0% w/v tapioca starch; 5.0 g gelatin; and 15.0 g agar.

Keywords: optimization, L-asparaginase, *Bacillus*, media

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L-asparaginase (EC 3.5.1.1) is the enzyme specifically catalyzing L-asparagine conversion to L-aspartate and ammonia [1] and playing important roles in the metabolism of all living organisms, as well as in pharmacology [2]. Treatment of cancer, especially acute lymphocytic leukemia, is the main potential therapeutic application of L-asparaginases. Tumor cells are not capable of synthesizing the normally nonessential amino acid, L-asparagine. Hence, they require high amount of asparagine. These leukemic cells highly depend on the circulating L-asparagine. Asparaginase, however, catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine, which leads to cell death [3–4]. On the other hand, the functioning of normal cells, which are able to produce sufficient amounts of L-asparagine, would not get affected by asparaginase. The enzyme is administered intravenously as it can provide a situation of fatal starvation for tumor calls by declining the concentration of free L-asparagine amino acids with relatively low side effects [5]. Numerous studies have been conducted on production of L-asparaginase by a great variety of microorganisms such as *Escherichia coli* [6, 7], *Erwinia carotovora* [8, 9], *Thermus thermophilus* [10], and *Enterobacter aerogenes* [11].

Nevertheless, since none of the known L-asparaginases are free of side effects, the need for discovering new enzymes with more desirable serological properties and less or no side effects still exists. The L-asparaginase isolated from *E. coli* and *Erwinia carotovora* is now being used in the treatment of acute lymphoblastic leukemia [12–14]. Additionally, different types of L-asparaginase have been used in the treatment of acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, Hodgkin disease, lymphosarcoma treatment, reticulosarcoma, melanosarcoma [13, 15], bovine lymphomosarcoma [16], and pancreatic carcinoma [17].

Screening and selection of the optimum concentration of medium components are very important to determine the overall economic feasibility of the production process. Nonetheless, as each organism has its own special conditions for maximum enzyme production, no defined medium has been established to optimize the production of L-asparaginase by various bacteria.

The goal of the present work was therefore to optimize the culture media for L-asparaginase production by newly isolated bacterium, *Bacillus* sp. GH5.

MATERIALS AND METHODS

Microorganism. The newly isolated L-asparaginase-producing bacterium, *Bacillus* sp. GH5, was

[1] The article is published in the original.

[2] Corresponding author; e-mail: s.gholami@zoho.com

used in the present study. The culture was grown on slants of modified M9 medium at 37°C for 24 h, subcultured at monthly intervals and stored in a refrigerator.

Preparation of spore suspensions. Spore suspension was prepared as an extra step to make sure that non-spore-forming bacteria (e.g. possible mutants) would not be selected, as they are not desirable in industry. Preparation of spore suspensions was carried out by adding 5 mL of 24 h old suspension—washed from a slant culture using sterile physiological saline—to 45 mL of sterile inoculum medium in 250 mL Erlenmeyer flasks.

The composition of inoculum medium was the following (g/L): $Na_2HPO_4 \cdot 2H_2O$, 6; KH_2PO_4, 3; NaCl 0.5; 1 M $MgSO_4 \cdot 7H_2O$, 2 mL; 0.1 M $CaCl_2 \cdot 2H_2O$, 1 mL; 20% glucose stock solution, 10 mL (pH 7.0).

Shake flask fermentation. After adding 5 mL of inoculum (10% vol/vol) to a 250-mL Erlenmeyer flask containing 45 mL of production medium, incubation of the flasks was carried out at 28°C on a rotary incubator shaker for 24 h (120 rpm). After fermentation, 5 mL broth was collected and centrifuged at 4000 rpm for 10 min and the assay of L-asparaginase activity was conducted. The production medium was prepared by adding 5 g/L L-asparagine to inoculum medium.

Protein assay. Determination of protein content was conducted according to a modified Lowry's method [18].

Enzyme assay. The activity of extracellular L-asparaginase was assayed by the direct Nesslerization method on collected supernatant from the late exponential phase [19]. International unit IU of L-asparaginase was calculated from the amount of enzyme that liberates 1 micromole of ammonia in 1 min at 37°C [20].

Screening of nutrients using the Plackett–Burman design. In order to screen the production of L-asparaginase by *Bacillus* sp. GH5 using the Plackett–Burman design, eleven sources of inorganic nitrogen and amino acids, as well as fifteen sources of carbon, organic nitrogen, and mineral salts were selected. In this design, the rows represent the variables (nutrients) and the columns represent the combinations. In addition, $4n$ (a multiple of four) experiments are required for screening $4n - 1$ components [21].

All the ingredients were divided into two levels (lower and higher). The lower level in the design is represented as "–" and the higher level as "+". The effect of an ingredient on the growth of the organism or yield of the enzymes was determined based on the *t*-value (main effect) calculated from the experimental result [22]. The value of an ingredient was calculated using the following equation: *t*-value or main effect of an ingredient X = (average of sum of the enzyme activities where the ingredients are "+") – (average of sum of the enzyme activities where the ingredient are "–"). The nutrients were ranked based on their *t*-values and those with the highest *t*-value was considered presenting the most effect and ranked one [23].

RESULTS AND DISCUSSION

Screening by the Plackett–Burman design showed that the components of the medium had a significant effect on L-asparaginase production by *Bacillus* sp. GH5. A total of 67 nutrients including 11 inorganic nitrogen sources and amino acid sources, as well as 15 carbon, nitrogen, and mineral salt sources were screened at this step. Plackett–Burman design which is a statistical methodology in the form of an orthogonal matrix, made it possible to monitor up to "$n - 1$" variables in only "n" experiments. To evaluate the positive or negative effect of each nutrient on the enzyme production, the absence or presence of the nutrients were observed.

Adjusting the concentrations of each nutrient was conducted empirically, based on the literature survey. Tables 1–5 present the list of nutrients with their respective concentrations.

Although L-asparaginase production and purification methods have been developed for many microorganisms such as *E. coli* [6] and *Erwinia cartovora* [8], still many potent bacteria that are able to produce a more desirable L-asparaginase remain uninvestigated. Therefore, production of L-asparaginase by other bacteria should be explored.

Moreover, medium factors can be manipulated in order to enhance the production of L-asparaginase. In the previous studies, L-asparaginase production by *Serratia marcescens* increased in the presence of yeast extract, lactose, and fructose while glucose decreased the enzyme production [24]. Likewise, enzyme production by *Erwinia aroideae* was enhanced by lactose and was repressed by glucose [25]. Moreover, yeast extract supported cell growth and enzyme production in *Vibrio succinogenes* [26]. In mutants of *Serratia marcesens*, namely mutants 933 and WF, enzyme production was improved by yeast extract. Whereas glucose and sucrose stimulated enzyme production in mutant 933, lactose inhibited enzyme production in mutant WF [27].

As each organism has its own special conditions for maximum enzyme production, no defined medium has been established to optimize the production of L-asparaginase for various bacteria. A disadvantage of single variable optimization methods is that they can lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked [28].

On the other hand, statistical experimental designs have been used for many decades and can be adopted at several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response [29–31]. A statistical approach has been employed in the present study for which a Plackett–Burman design is used for identify-

Table 1. Plackett–Burman design for the screening of fifteen carbon sources (mg/mL) $N = 16$

Dextrose	Lactose	Dextrin white	Starch potato	Starch soluble	Starch maize	Starch tapioca	Starch pharma	Maltose	Mannitol	Xylose	Fructose	Galactose	Sucrose	Cellulose	Yield, IU/mL
1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	35.50
1	1	1	0	1	0	1	1	0	0	1	0	0	0	1	23.25
1	1	0	1	0	1	1	0	0	1	0	0	0	1	1	41.75
1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	31.50
0	1	0	1	1	0	0	1	0	0	0	1	1	1	1	24.25
1	0	1	1	0	0	1	0	0	0	1	1	1	1	0	41.50
0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	35.25
1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	32.25
1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	34.50
0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	83.25
0	1	0	0	0	1	1	1	1	0	1	0	1	1	0	39.75
1	0	0	0	1	1	1	1	0	1	0	1	1	0	0	41.50
0	0	0	1	1	1	1	0	1	0	1	1	0	0	1	34.50
0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	30.75
0	1	1	1	1	0	1	0	1	1	0	0	1	0	0	35.25
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	31.25

Table 2. Plackett–Burman design for the screening of fifteen organic nitrogen sources (mg/mL) $N = 16$

Soya bean meal	Tryptone	Gelatin	Yeast extract	Soya peptone	Yeast nitrogen base	Casein	Meat extract	Peptone (B*)	Casamino acids	Malt extract	Beef extract	Urea	Albumin	Peptone (F**)	Yield, IU/mL
1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	32.75
1	1	1	0	1	0	1	1	0	0	1	0	0	0	1	30.25
1	1	0	1	0	1	1	0	0	1	0	0	0	1	1	20.25
1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	22.25
0	1	0	1	1	0	0	1	0	0	0	1	1	1	1	20.75
1	0	1	1	0	0	1	0	0	0	1	1	1	1	0	45.75
0	1	1	0	0	1	0	0	0	1	1	1	1	0	1	21.25
1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	22.75
1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	21.25
0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	25.00
0	1	0	0	0	1	1	1	1	0	1	0	1	1	0	23.25
1	0	0	0	1	1	1	1	0	1	0	1	1	0	0	22.25
0	0	0	1	1	1	1	0	1	0	1	1	0	0	1	35.75
0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	32.25
0	1	1	1	1	0	1	0	1	1	0	0	1	0	0	37.75
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32.25

Peptone (B*): Bacto^{TM}. Peptone (F**): Fisher Scientific.

Table 3. Plackett–Burman design for the screening of fifteen mineral salts (0.05 mg/mL) $N = 16$

$CaCl_2$	$CaCO_3$	K_2HPO_4	KH_2PO_4	$MnCl_2$	NaCl	KCl	Na_2HPO_4	Na_2SO_4	$MgCl_2$	$MnSO_4$	$NaHPO_4$	$MgSO_4$	$Fe(NH_4)SO_4$	$ZnSO_4$	Yield, IU/mL
0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	34.75
0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	32.25
0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	23.5
0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	17.75
0	0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	25.75
0.05	0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	27.5
0	0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	31.25
0.05	0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	28.00
0.05	0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	25.25
0	0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	21.75
0	0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	23.25
0.05	0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	26.25
0	0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	20.75
0	0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	23.5
0	0.05	0.05	0.05	0.05	0	0.05	0	0.05	0.05	0	0	0.05	0	0	25.75
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	31.25

Table 4. Plackett–Burman design for the screening of eleven inorganic nitrogen sources (0.4 mg/mL) $N = 12$

Ing. no. of experiments	$NH_4H_2PO_4$	$(NH_4)_2HPO_4$	NH_4Cl	NH_4NO_3	Amm. oxalate	Amm. acetate	Amm. sulfate	$NaNO_3$	KNO_3	Ferric amm. sulfate	Amm. hydroxide	Yield, IU/mL
1	0.4	0.4	0	0.4	0.4	0.4	0	0	0	0.4	0	30.25
2	0.4	0	0.4	0.4	0.4	0	0	0	0.4	0	0.4	26.25
3	0	0.4	0.4	0.4	0	0	0	0.4	0	0.4	0.4	28.05
4	0.4	0.4	0.4	0	0	0	0.4	0	0.4	0.4	0	23.25
5	0.4	0.4	0	0	0	0.4	0	0.4	0.4	0	0.4	27.25
6	0.4	0	0	0	0.4	0	0.4	0.4	0	0.4	0.4	33.05
7	0	0	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	36.25
8	0	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	25.25
9	0	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	0	28.25
10	0.4	0	0.4	0.4	0	0.4	0.4	0.4	0	0	0	28.05
11	0	0.4	0.4	0	0.4	0.4	0.4	0	0	0	0.4	34.25
12	0	0	0	0	0	0	0	0	0	0	0	31.05

Table 5. Plackett–Burman design for the screening of eleven different amino acids (0.1 mg/mL) $N = 12$

Ing. no. of experiments	D-Alanine	L-Cystine	L-Alanine	L-Cysteine	L-Lysine	L-Asparagine	D-Arginine	L-Arginine	L-Histidine	L-Glutamic acid	L-Tryptophan	Yield, IU/mL
1	1	1	0	1	1	1	0	0	0	1	0	23.75
2	1	0	1	1	1	0	0	0	1	0	1	21.25
3	0	1	1	1	0	0	0	1	0	1	1	20.25
4	1	1	1	0	0	0	1	0	1	1	0	27.75
5	1	1	0	0	0	1	0	1	1	0	1	32.75
6	1	0	0	0	1	0	1	1	0	1	1	42.25
7	0	0	0	1	0	1	1	0	1	1	1	35.75
8	0	0	1	0	1	1	0	1	1	1	0	31.25
9	0	1	0	1	1	0	1	1	1	0	0	28.25
10	1	0	1	1	0	1	1	1	0	0	0	37.75
11	0	1	1	0	1	1	1	0	0	0	1	30.25
12	0	0	0	0	0	0	0	0	0	0	0	31.25

ing significant variables influencing L-asparaginase production by *Bacillus* sp. GH5. Identification of the most effective nutrients was performed after applying the obtained data to statistical analysis.

The regression coefficient values or the *t*-value obtained from analysis of the experimental results was used for ranking the nutrients. The effectiveness of the nutrients in L-asparaginase production was evaluated based on the highest regression coefficient or *t*-value. According to Figs. 1–5, tapioca starch, gelatin, ammonium oxalate, $CaCO_3$, and L-asparagine were the most important sources for carbon, organic nitrogen, inorganic nitrogen, mineral salt, and amino acids respectively. The composition of the optimized media for 1 L was the following: 5.0 g L-asparagine; 0.5 g $MgSO_4 \cdot 7H_2O$; 6.0 g $NaHPO_4 \cdot 2H_2O$; 3.0 g

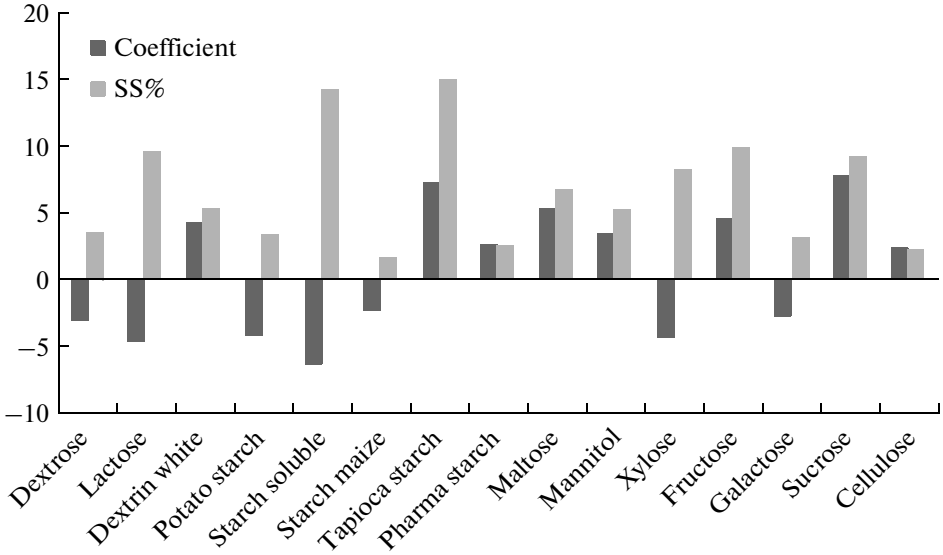

Fig. 1. Effect of fifteen different carbon sources (dextrose, lactose, dextrin, white starch, potato starch, soluble starch, maize starch, tapioca starch, pharma maltose, mannitol, xylose, fructose, galactose, sucrose, and cellulose) on the enzyme production.

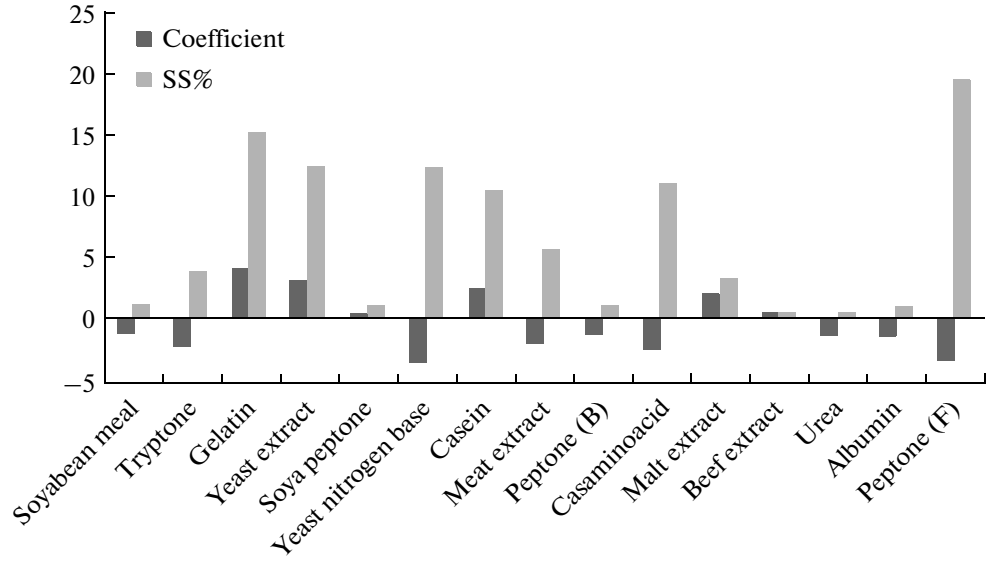

Fig. 2. Effect of fifteen different organic nitrogen sources (soyabean meal, tryptone, gelatin, yeast extract, soya peptone, yeast nitrogen base, casein, meat extract, peptone (B), casaminoacids, malt extract, beef extract, urea, albumin, peptone (F)) on the enzyme production.

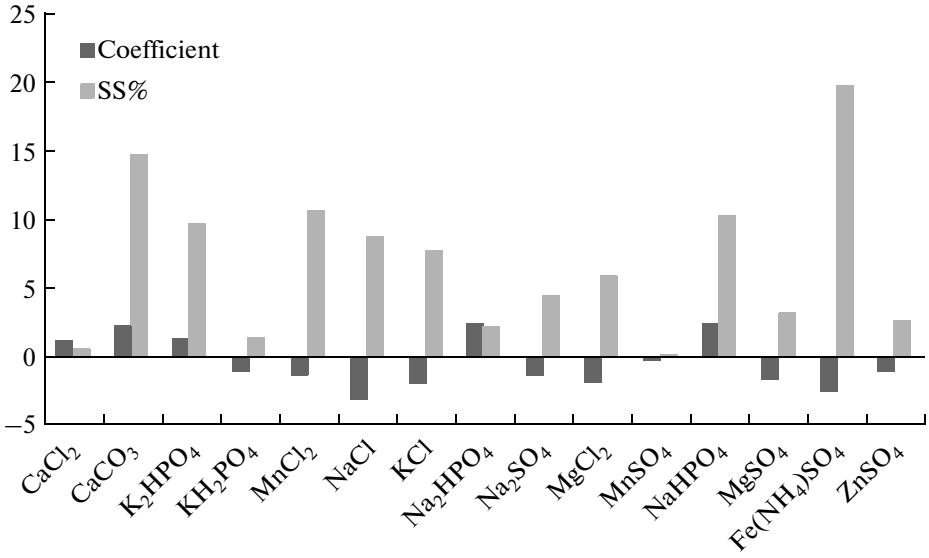

Fig. 3. Effect of fifteen different minerals ($CaCl_2$, $CaCO_3$, K_2HPO_4, KH_2PO_4, $MnCl_2$, NaCl, KCl, Na_2HPO_4, Na_2SO_4, $MgCl_2$, $MnSO_4$, Na_2HPO_4, $MgSO_4$, $Fe(NH_4)SO_4$, and $ZnSO_4$) on the enzyme production.

$(NH_4)_2C_2O_4$; 0.5 g $CaCO_3$; 0.014 g $CaCl_2 \cdot 2H_2O$; 2.0% wt/vol tapioca starch; 5.0 g gelatin; and 15.0 g agar.

A similar study investigated the possible effects of media composition on asparaginase activity using five different media [32]. The results showed that using the optimized medium resulted in a significantly higher enzyme production (more than threefold, from 9.569 to 45.374 IU/mL). Two other recent studies used the Plackett–Burman design in order to obtain the optimized medium for asparaginase production by *Aspergillus terreus* [33] and *Pectobacterium carotovorum* [34]. The method proved efficient in maximizing the enzyme production up to 40.86 IU/mL for *A. terreus* and 3.25 U/mL for *P. carotovorum*. Similarly, in our study the maximum L-asparaginase activity occurred in the optimized media at 37°C and pH 7.0 and was equal to 57.12 IU/mL. L-asparaginase

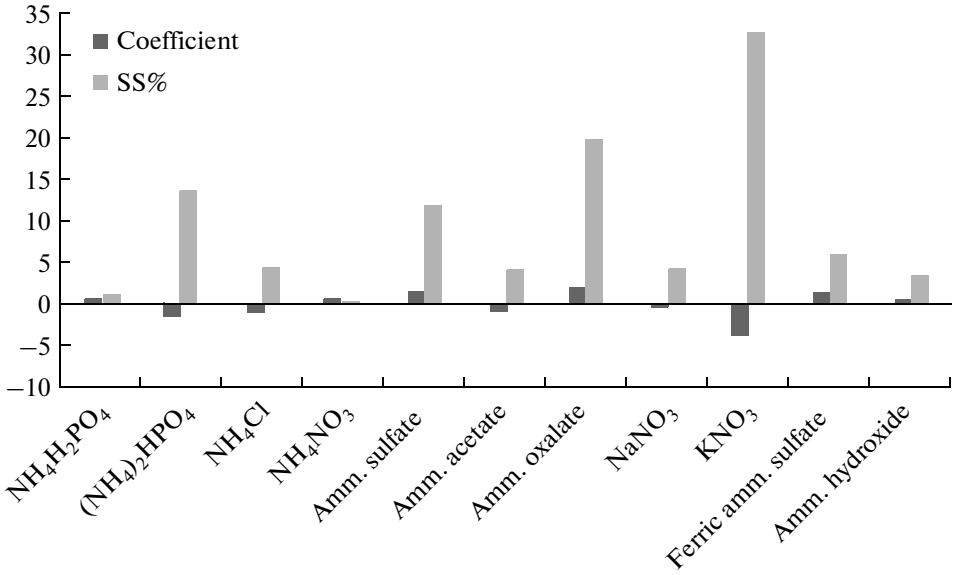

Fig. 4. Effect of eleven different inorganic nitrogen sources ($NH_4H_2PO_4$, $(NH_4)_2HPO_4$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, ammonium acetate, ammonium oxalate, $NaNO_3$, KNO_3, ferric amm. sulfate, and NH_4OH) on the enzyme production.

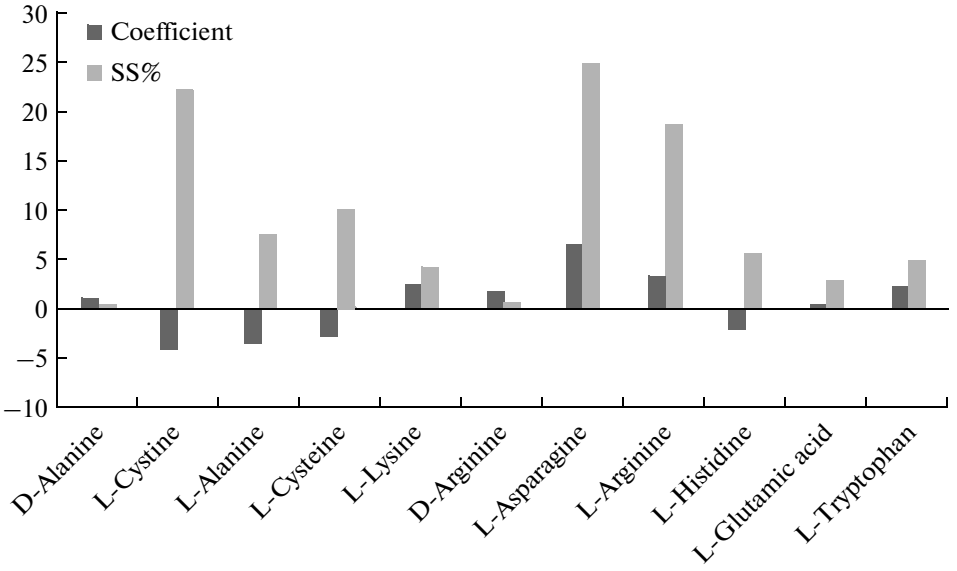

Fig. 5. Effect of eleven different amino acids (D-alanine, L-cystine, L-alanine, L-cysteine, L-lysine, D-arginine, L-asparagine, L-arginine, L-histidine, L-glutamic acid, L-tryptophan) on the enzyme production.

activity in M9 medium supplemented with asparagine under the same conditions was 14.3 IU/mL.

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