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Amino acids requirements for growth and toxin production by *Lysinibacillus sphaericus*

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ABSTRACT

Growth and mosquitocidal activity of *Lysinibacillus sphaericus* 9B24 was studied in defined medium supplemented with amino acids. It was found that L-glutamate, L-arginine, and L-proline enhanced the sporulation and mosquitocidal activity of the tested culture against *Culex pipiens* larvae. Different concentrations of these three amino acids were tested for sporulation and toxin formation by *Lysinibacillus sphaericus* 9B24 in comparison with *Lysinibacillus sphaericus* 2362. Optimum concentrations were 0.5-0.75% for L-arginine and 0.75% for L-glutamate and L-proline. All tested mixtures of these amino acids enhanced the sporulation and mosquitocidal toxicity of both cultures. L-arginine- L-proline - L-glutamate mixture at their optimum concentrations was the most mosquitocidal enhancer for *Lysinibacillus sphaericus* 2362 (LC_{50} 1.5×10^{-5}) however, mixture of L-proline- L-glutamate was the optimum for *Lysinibacillus sphaericus* 9B24 (LC_{50} 1.66×10^{-5}). It was concluded that the wastes which contain high quantities of L-arginine, L-proline and L-glutamate could be used as media for cost effective production of *Lysinibacillus sphaericus.*

KEYWORDS

Lysinibacillus sphaericus;
Amino acids;
L-glutamate;
L-arginine;L
L-proline;
Culex pipiens.

INTRODUCTION

It has been established that *Lysinibacillus sphaericus* (Ls) cannot use carbohydrates as a source of carbon and energy[1]. Accordingly, the carbon source for growth and energy production should be provided by protein–rich medium components, especially partially hydrolyzed proteins or amino acids[2]. It was reported that many strains of Ls grew well and sporulate on a single amino acid medium or on a mixture of several amino acids[3]. It was proposed that these amino acids probably serve as primary carbon sources for growth in protein-rich media[4]. The same conclusion was confirmed by Lacey[5] and Klein et al[6]. They found that glutamic acid, lysine, glycine and valine promoted growth and toxin production of Ls strains (Ls 2362 and Ls 1593). Moreover, addition of proline to glutamic acid enhanced the toxin production of Ls 2362. Shevtsov et al.[7] studied the requirements for amino acids and other growth and spore germination factors of 16 different strains of Ls. They found that the majority of strains required arginine, glutamic acid, methion-

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ine, threonine, serine, alanine, and lysine but did not utilize phenylalanine or proline. They also reported that the most effective inducers of spore germination were arginine, methionine, and glutamic acid. It was reported that Ls 2362 grew with increasing doubling times on histidine and arginine as carbon and energy sources[8].

Our team isolated a highly mosquitocidal strain of Ls namely Ls 9B24[9]. The aim of the present study was to study the requirement of this strain to amino acids in the medium for sporulation and mosquitocidal toxin production. This will help in choosing the suitable wastes which contain these amino acids for feasible and cost effective production of Ls.

EXPERIMENTAL

Micro-organisms and inoculum preparation

The Egyptian isolate Ls 9B24 and the standard strain Ls 2362 were used in this study. Ls 9B24 was isolated from Alexandria governorate and showed high potency as larvicidal of *Culex pipiens*[9]. The international strain, Ls 2362 was obtained from Prof. Dr. Fergus G. Priest, Heriot –Watt University, United Kingdom.

Bacterial cultures were inoculated to 25 ml of NYSM medium[10] and incubated for 3 days at 30°C under shaking conditions.

Selective utilization of different amino acids for growth and toxin production by *Ls* 9B24

A group of amino acids were tested to study their influence on the growth and toxin production of Ls 9B24. The amino acids were used as supplements to modified BATS medium at a final concentration of 0.5 % (w/v). These amino acids were L-aspartic acid, L-asparagine, L-glutamate, L-glutamine L-histidine, L-arginine, L-lysine, L-alanine, L-leucine, L-serine, L-threonine, L-proline, L-hydroxyproline, L-glycine, L-phenylalanine, L-tyrosine, L-tryptophan, L-cystine, L-cysteine and L-methionine.

Different concentrations (0.125% - 1%) of selected amino acids which enhanced the toxin production were further tested for enhancing growth and toxicity with of Ls 9B24 in comparison with Ls 2362.

Mixtures of these amino acids at their optimum concentrations were tested for its effect on sporulation and toxicity of both tested organisms.

Modified BATS medium contains/l, K_2HPO_4, 5.57 g; KH_2PO_4, 2.40 g; $MgSO_4.7H_2O$, 50 mg; $MnCl_2.4H_2O$, 4 mg; $FeSO_4.7H_2O$, 2.80 mg; $CaCl_2.2H_2O$, 1.50 mg; Thiamine, 20 mg; Biotin, 2 µg and Yeast extract, 3 mg. The biotin, thiamine and yeast extract were prepared as a filter – sterilized stock solution. The Mg^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+} salts were prepared as an acidified (0.03% v/v concentration of H_2SO_4), autoclaved stock solution. These two stock solutions were added to the autoclaved phosphate salts mixture.

Toxicity tests

Bioassay of isolated bacterial cultures was adopted from Priest and Yousten[11] with slight modifications. Toxicity was determined with laboratory reared second instar larvae of C*ulex pipiens.* Bacterial dilutions of final whole culture were placed into 100 ml beakers in duplicate along with 10 second instar *Culex pipiens* larvae. About 10 mg of ground fishmeal was added to each cup. The beakers were kept at 26±2ºC with a 10h light/14h dark cycle. The mortality percentage was recorded by counting the number of living larvae and adopting Abbot's formula[12].

Statistical analysis

Data obtained from this study were statistically analyzed according to SPSS system[13] using one-way analysis and the Duncan's multiple range test[14] to determine the significance between means. Data were expressed as mean values ± standard errors of three samples for both spore count and mortality percent. LC_{50} calculations were done using proban software[15].

RESULTS AND DISCUSSION

Utilization of different amino acids by Ls 9B24 for growth and toxin production

L-asparagine, L-glutamate, L-histidine, L-arginine, L-serine, L-proline and L-hydroxyproline supported good growth as determined by optical density at 650 nm, while other amino acids showed low optical density as L-aspartic acid, L-glutamine, L-lysine, L-alanine, L-leucine, L-threonine, L-glycine, L-phenylalanine, L-tryptophan and L-methionine (TABLE 1). For

L-tyrosine, L-cystine and L-cysteine the optical density was not measured as they did not dissolve in the medium.

TABLE 1 : Effect of different amino acids incorporated in modified BATS medium, on growth and mosquitocidal activity of Ls 9B24

Amino acids tested	O.D $_{650nm}$	Mortality % at $5x10^{-5}$ after 48 h
Acidic amino acids and amides		
L-aspartic acid	0.15	0 e
L-asparagine	1.38	40 c
L- glutamate	1.87	100 a
L-glutamine	0.20	0 e
Basic amino acids		
L-histidine	1.30	41.67±4.41 c
L-arginine	1.83	100 a
L-lysine	0.28	0 e
Neutral amino acids		
L-alanine	0.38	0 e
L-leucine	0.40	13.33±3.33 e
L-serine	1.52	31.37±1.67 d
L-threonine	0.61	0 e
L-proline	1.45	91.67±6 b
L-hydroxyproline	1.31	86.67±7.26 b
L-glycine	0.63	25±2.89 d
Aromatic amino acids		
L-phenylalanine	0.14	5±2.89 e
L-tyrosine	Not determined	0 e
L-tryptophan	0.117	0 e
Sulfur amino acids		
L-cystine	Not determined	0e
L-cysteine	Not determined	0 e
L-methionine	0.16	0 e
Control (no amino acid)	0.17	0 e

Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P ≤ 0.05

The mosquitocidal activity was determined at $5x10^{-5}$ dilution against second instar *Culex pipiens* larvae. The results showed that the amino acids which enhanced the obtained toxicity by tested culture were L-glutamate, L-arginine, L-proline and L-hydroxyproline. Other amino acids gave lower or no mortality at tested dilution.

It was reported that glutamic acid, lysine, glycine and valine promoted growth and toxin production of Ls 2362 and Ls 1593[5,6]. Amino acid analysis of NYSM broth after growth for seven hours by Ls 1593 revealed that glutamic acid and amino acids catabolized via glutamate (proline, arginine and histidine) as well as aspartic acid and glycine were readily utilized as reported by Yousten[4].

Shevtsov et al.[7] found that the majority of strains required arginine, glutamic acid, methionine, threonine, serine, alanine and lysine but did not utilize phenylalanine or proline. Also, it was reported that Ls 2362 grew with increasing doubling times on acetate, gluconate, histidine, arginine and succinate as carbon and energy sources[8].

Based upon those findings, three of these amino acids (L-glutamate, L-arginine and L-proline) were selected for studying their effect on sporulation and toxin production of Ls 9B24 as compared to Ls 2362 using different concentrations (0.125% - 1%) in modified BATS medium.

Effect of L-glutamate concentration on sporulation and mosquitocidal activity of Ls

Maximum sporulation of Ls 2362 was at 0.5-1% L-glutamate however for Ls 9B24 was 0.75-1% as shown in TABLE 2. More or less L-glutamate leads to lower sporulation in both cultures.

The mosquitocidal activity against *Culex pipiens* larvae was increased with increasing L-glutamate concentration with optimum concentration at 0.75-1% for both cultures. Sadek[16] found that L-glutamate enhanced the growth and sporulation of *Bacillus thuringiensis aizawai*. It was reported that the importance of L-glutamate in the central metabolism of bacilli is not only due to being a precursor for biosynthesis of a group of amino acids but it is also of great importance in various processes involving the transfer of nitrogen to other metabolites through reactions catalyzed by glutamate oxaloacetate transaminase, glutamate-pyruvate transaminase and glutamate dehydrogenase[17].

Effect of L-arginine concentration on sporulation and mosquitocidal activity of Ls

The optimum concentration of L-arginine for the highest sporulation was 0.75-1% for Ls 2362 and 1% for Ls 9B24 as shown in TABLE 3,.

On the other hand, optimum L-arginine concentra-

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TABLE 2 : Effect of L-glutamate concentration incorporated in modified BATS medium on sporulation and toxicity of Ls 2362 and Ls 9B24

L- glutamate concentration (%)	Sporulation yield (CFU x10^6/ ml)		Mortality % at 5x10^{-5} after 48h	
	Ls 2362	Ls 9B24	Ls 2362	Ls 9B24
0.125	12±1.15 c	18.33±1.20 c	10±2.89 d	0 d
0.25	23±3.51 b	29±3.79 b	18±4.41 c	31.67±1.67 c
0.5	43.67±1.86 a	26.67±1.33 b	80±2.89 b	75±2.88 b
0.75	44±2.65 a	45±2.89 a	93.33±1.67 a	80±5.77 ab
1	36±3.6 a	44.33±0.88 a	96.67±1.67 a	85±2.89 a
Control	3.33±0.88 d	4.33±1.20 d	0 e	0 d

Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P≤ 0.05

TABLE 3 : Effect of L-arginine concentration incorporated in modified BATS medium on sporulation and toxicity of Ls 2362 and Ls 9B24

Arginine concentration (%)	Sporulation yield (CFU x10^6/ ml)		Mortality% at 5x10^{-5} after 48h	
	Ls 2362	Ls 9B24	Ls 2362	Ls 9B24
0.125	31±0.58 d	23.33±0.88 c	0 c	0 c
0.25	39±4.7 c	38.67±0.67 b	51.67±3.33 b	60±5.78 b
0.5	40±2.9 b	42±1.53 b	55±5 b	66.60±4.41 b
0.75	45±2.51 ab	41.67±1.67 b	96.67±1.67 a	86.67±1.67 a
1	49±0.58 a	70±1.15 a	93.33±1.67 a	83.33±1.67 a
Control	4.43±0.29 e	3 d	0 c	0 c

Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P≤ 0.05

TABLE 4 : Effect of L-proline concentration incorporated in modified BATS medium on sporulation and toxicity of Ls 2362 and Ls 9B24

L-proline concentration (%)	Sporulation yield (CFU x10^6/ ml)		Mortality % at 5x10^{-5} after 48h	
	Ls 2362	Ls 9B24	Ls 2362	Ls 9B24
0.125	20±0.58 b	15±0.58 c	0 c	0 c
0.25	24.67±2.03 b	20.67±3.67 c	0 c	41.67±1.67 b
0.5	28±1.53 b	21.67±1.67 c	73.33±3.33 b	91.67±4.41 a
0.75	31.67±4.41 b	51.67±4.4 b	93.33±1.67 a	91.67±4.41 a
1	63.33±8.82 a	62.67±1.45 a	93.33±1.67 a	93.33±1.67 a
Control	5.10±0.49 c	3 d	0 c	0 c

Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P≤ 0.05

tion for the highest mosquitocidal activity was 0.75 - 1% for both cultures.

It was reported that arginine is required for both growth and sporulation of Ls[7].

Effect of L-proline concentration on sporulation and mosquitocidal activity

The sporulation yield was stable for Ls 2362 at proline concentrations 0.125-0.75% then reached the maximum at 1% as shown in TABLE 4. Also for Ls 9B24 the sporulation was stable at proline concentration between 0.125-0.5% after that it gradually increased reached its maximum at 1%.

The mosquitocidal activity also increased with increasing the proline concentration until it reached the maximum value at a concentration of 0.75-1% for Ls 2362 and 0.5-1% for Ls 9B24.

Effect of mixtures of amino acids on sporulation and mosquitocidal activity

TABLE 5 : Effect of mixtures of the selected amino acids on porulation and mosquitocidal activity of Ls 2362 and Ls 9B24

Amino acid (concentration (%))	Sporulation yield (CFU x10^6/ ml)		Mortality % at 1.2x10^{-5} after 48h	
	Ls 2362	Ls 9B24	Ls 2362	Ls 9B24
L-proline (0.5)	32±1.52 d	26.67±0.88 c	21.67±1.67 c	16.67±1.67 de
L-arginine (0.75)	33.67±1.86 cd	35.67±4.70 bc	23.33±1.67 c	15±2.89 e
L-sodium glutamate (0.75)	32.67±4.06 d	35.67±2.33 bc	10±2.89 d	8.33±1.67 f
L-proline (0.5) + L-arginine (0.75)	54±7.02 a	48±1.76 a	35±2.89 b	23.33±1.67 bc
L-proline (0.5) + L-sodium glutamate (0.75)	46±3.06 abc	53±4.58 a	31.67±1.67 b	21.67±1.67 cd
L-arginine (0.75) + L-sodium glutamate (0.75)	50±5.29 ab	46.33±4.91 ab	21.67±1.67 c	43.33±3.33 a
L-proline (0.5) + L- rginine (0.75) + L-odium glutamate (0.75)	39±3.79 bcd	53.33±3.53 a	43.33±1.67 a	28.33±1.67 b

Mortality % is expressed as mean value ± standard error. Values for each treatment per tested organism followed by different letters are significantly different at P≤ 0.05

TABLE 6 : LC_{50} and LC_{90} of Ls cultures grown in modified BATS medium incorporated with some amino acids

Amino acids incorporated in modified BATS medium	Ls 2362		Ls 9B24	
	LC_{50} x10^{-5} (95% fiducial limits)	LC_{90} x10^{-5} (95% fiducial limit)	LC_{50} x10^{-5} (95% fiducial limits)	LC_{90} x10^{-5} (95% fiducial limit)
L- arginine	2.76 (1.97-3.37)	6.02 (4.77-9.86)	3.12 (2.82-3.43)	6.84 (5.88-8.40)
L- proline	3.10 (2.51-3.57)	7.45 (5.90-11.51)	2.61 (2.37-2.86)	5.77 (5.10-6.76)
L-sodium glutamate	2.96 (2.51-3.39)	5.27 (4.54-6.52)	2.56 (2.17-3.01)	6.13 (5.14-7.52)
L-arginine + L- sodium glutamate	1.82 (1.46-2.14)	4.75 (3.95-6.23)	2.42 (1.88-2.88)	5.47 (4.48-7.67)
L-proline + L- sodium glutamate	2.26 (1.90-2.60)	4.89 (4.07-6.53)	1.66 (1.25-2.05)	5.17 (4.14-7.47)
L-proline + L-arginine	1.84 (1.66-2.01)	4.37 (3.87-5.13)	2.25 (1.81-2.66)	5.01 (4.08-7.07)
L-arginine + L- proline + L-sodium glutamate	1.50 (1.34-1.66)	4.02 (3.47-4.87)	1.69 (1.49-1.91)	4.26 (3.60-5.30)

The optimum concentration of each of the three amino acids namely L-glutamate, L-arginine and L-proline were selected and used in mixtures to test their effect on sporulation and toxicity of Ls.

TABLE 5 indicates that there is an increase in sporulation titers for the three amino acids mixtures than that of each of the individual amino acids for both organisms.

Also as regards to the mosquitocidal activity (TABLEs 5&6) mixtures of amino acids have exhibited higher activity than that of individual amino acids. For Ls 2362, the maximum toxicity was obtained with the mixtures of three selected amino acids (LC_{50}, 1.5×10^{-5}). However, mixture of L-glutamate and L-arginine enhanced the toxicity produced by Ls 9B24 (LC_{50}, 1.66×10^{-5}).

In agreement with these data Lacey[5] and Klein et al.[6] reported that the addition of proline to glutamic acid enhanced the toxin production of Ls 2362. According to Zubary[18] the pathways of amino acids biosynthesis in microorganisms could be divided to six families depending on the main precursor from which they are derived. Glutamate family of amino acids comprises glutamate, glutamine, ornithine, arginine and proline.

The results in the present study confirm the unique position of L-glutamate family amino acids as indispensible key amino acids in the metabolic processes leading to sporulation and toxin production of Ls.

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