"Production of L-asparaginase by Streptomyces karatakensis and Streptomyces venzuelae"

by

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INTRODUCTION

Considerable attention to the enzyme L-asparaginase (L-asparagine amino hydrolase) has resulted from the discovery of its ability to inhibit growth of tumors in the mouse, rat and dog and to suppress human leukemias in clinical trials (CAPIZZI et al 1970). It has been suggested that the antitumor activity of this enzyme is due to the fact that a number of tumor cells responding to it lack adequate L-asparagine synthetase activity and require an exogenous supply of L-asparagine. Depletion of this amino acid by L-asparaginase in vitro or in vivo results in the death of these tumor cells.

A tumor inhibitory asparaginase, designated EC-2 and obtained from strains of E. coli was prepared on a large scale and has been used extensively for clinical trials and other investigations. Factors affecting the biosynthesis in flask cultures has been studied (ROBERTS et al 1968). A partially purified asparaginase from S.marcencens ATCC 60 was described (ROWLEY and WRISTON 1967). and fermentation conditions which produce high yields of asparaginase in shaken cultures was investigated (HEINEMANN and HOWARD 1969).

Actinomycetes has been neglected as a potential source of L-asparaginase. Recently a number of Streptomyces isolates obtained from the soil produced detectable amounts of this enzyme. (MOSTAFA 1977). The present investigation is carried to determine the growth conditions for two different streptomyces which produce high yield of asparaginase in surface cultures.

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Materials and Methods.

1. Microorganisms.

The two microorganisms used in this study were isolated from fertile Egyptian soil and one of them has been previously identified as a strain of Streptomyces karnatakensis (MOSTAFA 1977). The second (Streptomyces - 9) will be identified in this report. All the methods used for morphological and physiological studies were those of the International Streptomyces Project (I.S.P.) as described by SHIRLING and GOTTLEIB (1966). Direct mount of spores on collodiun films was examined by a transmission electron microscope (EM9S-2), The Electron Microscope Unit, Kuwait University) and electron photomicrograph was taken. Tentative identification was made mainly by using the key suggested by KUSTER (1972), however, BERGEY (1974) and SZABO (1975) were consulted.

L-asparaginase assay:

Mycellium matt obtained from a 6 - 8 days old culture and washed three times with distilled water was used as a source of enzyme. The standard L-asparaginase test system contained in a total volume of 2 ml : 20 μmoles. L-asparagine; 500 μmoles tris buffer pH 8.6; 100 mg (fresh wt) mycellium. The reaction was initiated by the addition of the substrate and the reaction mixture was incubated at 37°C for 1 hour after which the reaction was terminated by the addition of 0.5 ml of 1.5 M trichloroacetic acid. This was followed by filteration on Whatman no. 1 filter paper. The amount of ammonia liberated was then measured spectrophotometrically at 450 mμ by Nesslerization. For each enzyme assay two controls were always included, one with TCA denatured enzyme and the other without substrate. All enzyme assays were carried in triplicate and the average was recorded. The concentrations of ammonia in the test solutions were determined from a standard curve with ammonium sulphate as the source of dissolved ammonia

The rate of the assay reaction was determined to be linear with respect to enzyme concentration under the conditions and over the range of the enzyme assay. One international (I.U.) L-asparaginase unit is defined as the amount of enzyme which liberates 1 μmole of ammonia / minute at 37°C.

L-asparaginase production and the factors affecting it.

A number of liquid media were examined for their effect on L-asparaginase production these included nutrient broth, inorganic salts starch as described by KUSTER (1959), Czapek, and ISA liquid medium. The latter contained (g/L): K_2HPO_4, 1 ; $MgSO_4.7H_2O$, 0.5; NaCl, 1; trace salts solution (as described by KUSTER (1959), 1 ml; L-asparagine, 2.0; soluble starch 10.

The above ISA liquid medium was used as the basal salt medium for investigating various environmental and nutrirional factors affecting L-asparaginase production. Thus the effect of various carbon sources was examined by substituting starch in the above medium with one of the tested carbon sources namely, glycerol, glucose, fructose, sucrose, lactose and maltose. Various nitrogen sources were also investigated by substituting L-asparagine in the ISA medium with an equimollar amount of one of the following amino acids: isoleucine, leucine, arginine, Aspartic, Cystine, Methionine, histidine and glutamine. Moreover the effect of histidine, glutamine and aspartic when each of them was used in the ISA medium additional to asparagine was examined. Various starch and L-asparagine concentrations in the ISA medium were also investigated.

L-asparaginase produced during incubation at various temperatures was assessed by incubating inoculated ISA medium for 7 days at the specified temperature.

Static and shaken cultures were examined for their growth and L-asparaginase productivity at various ages. Growth was followed by dry weight determination.

In all cases a standard inoculum was used. The standard inoculum was prepared by suspending the sporulating growth on an agar slant of inorganic salts starch agar medium (SSA) in 3 ml of sterile water, this was transfered asceptically to a small screw cap sterile bottle containing few glass beads. The spore suspension is shaken vigorously for a few minutes with the glass beads to form homogenous suspension. This suspension was used for inoculation at a final concentration of 2%.

Results.

Identification of Streptomyces - 9.

Streptomyces-9 is a member of the grey series, is melanin positive, does not produce soluble pigments, mature spores were carried in straight or wavy chains (Plate 1) i.e. section rectiflexibles. Photoelectron micrograph revealed smooth spore surface (Plate 1). Spore chains are generally long, often with more than 50 spores per chain. This morphology is seen on oatmeal agar, salts starch agar and glycerol asparagine agar. Fragmentation of the substrate mycellium was sometimes noticed on glycerol asparagine agar medium. On agar media containing glucose the substrate mycellium showed a distinct reddish brown colour. Streptomyces-9 produced an antibiotic which was active against Gram positive (Bacillus subtilis) and Gram negative (Escherichia coli) organisms. Streptomyces-9 was able to utilize any of glucose, xylose, rhamnose and arabinose. Fructose was utilized poorly while inositol, sucrose, mannitol and raffinose were not utilized by this organism.

Comparing the above morphological and physiological characteristics with those used in the working key suggested by KUSTER 1976, Streptomyces-9 was identified as Streptomyces venezuelae. Furthermore, comparing all the present isolate characteristics with those of S. venezuelae as described by SHIRLING and GOTTLIEB, they were found identical.

Production of L-asparaginase by S.karnatakensis and S.venezuelae

Among all the tested media for L-asparaginase production both S.karnatakensis and S.venezuelae produced more enzymes when they were grown on the ISA liquid medium. Consequently this medium was used as the growth medium for investigating the various factors affecting L-asparaginase production.

Factors affecting L-asparaginase production:

Growth and enzyme production were greatly reduced to various degree when starch in the ISA liquid medium was substituted with any of the carbon sources examined. Consequently starch was used as the carbon source in the ISA medium for further investigation. Increasing concentrations (up to a certain extent) of starch in the ISA medium stimulated growth and enzyme productivity of both organisms (Table 1).

Both organisms produced more L-asparaginase when L-asparagine was used as the only added nitrogen source in the ISA medium than when equimollar amounts of any of the tested amino acids was used. Few of the tested amino acids e.g. aspartic, glutamic, cystine and tryptophan supported neither growth nor enzyme production (untabulated). Leucine and isoleucine gave poor growth while arginine supported good growth and the enzyme activity in that growth was about 50% that obtained when L-asparagine was used. The two organisms responded differently to the presence of histidine and glutamine in the growth

medium. Thus while histidine inhibited growth and enzyme production by S. karnatakensis, it stimulated S. venezuelae growth and enzyme production when it was present additional to asparagine. Glutamine stimulated enzyme productivity of both organisms when it was present additional to asparagine (Table 2).

Optimum L-asparaginase production by S. karnatakensis and S. venezuelae occured when the L-asparagine concentration in the ISA medium was 1 and 0.5% respectively (Fig.1). Optimum growth did not always coincide with optimum enzyme production (Fig.1).

28°C was found optimum for growth and enzyme production by both organisms. At temperature above 40°C both organisms were unable to show detectable growth or enzyme activity. At 15°C S. venezuelae but not S. karnatakensis showed some growth.

Both organisms showed their maximum enzyme production in shaken cultures after 48 hours of incubation at 28°C, older shaken cultures showed increased growth but declined enzyme productivity. Static cultures of S. karnatakensis and S. venezuelae showed their optimum enzyme productivity after 5 and 2 days respectively. (Table 3). Although S. venezuelae was able to grow and produce asparaginase at the tested pH values, optimum growth and enzyme production occured at pH 8.5 and 6.5 respectively (Fig. 2) S. karnatakensis showed its optimum growth and enzyme productivity at pH 7.4. Higher and lower pH values inhibited growth and enzyme production.

Discussion.

The results of this study indicate that the identity of the organism as well as the environmental factors are important for the biosynthesis of L-asparaginase. As with a number of other enzymes, formation of L-asparaginase is inhibited by the addition

of sugars particularly glucose (HEINEMANN and HOWARD 1969). The mechanism of this depressive effect is thought to result from the presence of glucose metabolic products (MOSES and PREVOST 1966). In the case of L-asparaginase biosynthesis the depressive effect of carbohydrates may be a function of their ability to lower the pH value of the growth medium (HEINEMANN and HOWARD, 1969). It is interesting to point out that starch proved to be the best carbon source for L-asparaginase production. The use of starch as a carbon source and consequently the production of α amylase has been linked with the biosynthesis of other enzymes e.g. lipase (ELWAN et al 1977). The results of the present investigation might add another evidence to the possible association of starch metabolism with the biosynthesis of other enzymes namely L-asparaginase. The depressive effect of high starch concentrations is probably due to an excess carbohydrate and/or of a low pH (WADE et al 1971). The latter is more likely in the present investigation. Thus S. venezuelae which exhibited its optimum enzyme productivity at pH 6.5 was also able to produce more enzyme at higher starch concentration (4%), and lower asparagine concentration (0.5%). On the other hand S.karnatak-ensis which showed an optimum pH of 8.5 required lower starch concentration (2%) and higher asparagine concentration (1%) for optimum enzyme productivity. The substrate of L-asparaginase stimulated its biosynthesis while L-aspartic which is the reaction product, inhibited the enzyme biosynthesis. This might be another example of feed-back mechanism, however, the effect of aspartic in lowering the pH of the medium and consequenrly inhibiting the enzyme biosynthesis and/or activity could not be ignored. The feed back mechanism might explain the depression of enzyme productivity of both organisms in static as well as in shaken cultures after a certain enzyme level has been attained. This enzyme level is reached after 48 hours of incubation of S. venezuelae in static or shaken cultures. S karnatakensis on the other hand reaches this enzyme level after 48 and 120 hours in shaken and static cultures

respectively. The high enzyme level in the medium results in the accumulation of aspartic which inhibits the biosynthesis of more enzyme upon further incubation. Aeration of the culture medium is stimulatory for the growth of both S. karnatakensis and S. venezuelae but not for the production of L-asparaginase. This is in accordance with the results of HEINEMANN and HOWARD (1969) who noticed a decreased enzyme synthesis by S. marcescens upon aeration. Moreover enhanced biosynthesis of L-asparaginase by E. coli under anaerobic conditions has been reported by CEDAR and SCHWARTZ (1968).

The information gained from the present investigation will make it possible to prepare uniform growth from S. karnatakensis and S. venezuelae which could be disrupted and the L-asparaginase can be purified and studied in the cell free extract.

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Summary

Production of L-asparaginase by two soil isolates identified as S. karnatakensis and S.venezuelae under different environmental and nutritional conditions was investigated. The presence of carbon sources other than starch in the growth medium or amino acids, other than L-asparagine inhibited the enzyme biosynthesis. L-aspartic inhibited growth and enzyme production due to a feed-back mechanism, and/or lowering the pH value. Both organisms were stimulated to produce more enzyme with increasing concentrations of starch and L-asparagine, however, the optimum starch and L-asparagine concentration depended on the tolerance of the organism to low and high pH respectively. Aeration stimulated growth but not enzyme production and both organisms produced more enzyme in static cultures than in shaken cultures.

Table 1

Starch Conc. %	Streptomyces karnatakensis			Streptomyces venezuelae		
	Dry wt mg /100 ml medium	Final pH	Enzyme conc. I.U. / gm dry wt.	Dry wt. mg /100 ml medium	Final pH	Enzyme conc. I.U. / gm dry wt.
0.1	060	8.0	61.6	020	8.6	10
0.5	165	7.9	62.5	080	8.3	20.83
2.0	200	7.5	70.83	240	5.7	38.83
4.0	120	7.2	0.66	220	5.7	55.83

Effect of starch conc. on L-asparaginase production by S.karnatakensis and S.venezuelae. Medium was ISA liquid medium with starch concentration as specified in the table. Incubation in 50 ml medium in 250 ml at 28°C for 6 days in static cultures. Enzyme assay was carried out as described in the text.

Table 2

Amino acid used	Streptomyces karnatakensis			Streptomyces venezuelae		
	Dry wt mgm / 100 ml medium	Enzyme conc I.U. / gm dry wt.	Final pH	Dry wt. mgm / 100ml medium	Enzyme conc. I.U. / gm dry wt.	Final pH
Asparagine	190	40.8	7.45	240	20.83	8.0
Histidine	-	-	5.8	255	8.33	6.8
Histidine + asparagine	-	-	5.8	271	35.00	8.14
Glutamine	138	20	6.18	021	5.00	7.0
Glutamine + asparagine	237	116.6	8.22	208	33.33	7.9
Aspartic	-	-	3.60	-	-	3.6
Aspartic + aspargine	-	-	3.70	-	-	3.7

(-) not carried out due to poor growth

L-asparaginase productivity of S. karnatakensis and S. venezuelae in the presence of different amino acids.

Medium was ISA liquid medium with .015 M of the specified amino acid as the nitrogen source. Incubation at 28°C for 6 days in static cultures. Enzyme assay was carried out as described in the text.

Table 3

Culture age (hrs)	Streptomyces karnatakensis						Streptomyces venezuelae					
	Static cultures			shaken cultures			static cultures			shaken cultures		
	dry wt. mgm /100 ml medium	enzyme conc. I.U./ gm dry wt.	Final pH	dry wt. mgm /100 ml medium	enzyme conc. I.U. / gm dry wt.	Final pH	dry wt. mgm /100 ml medium	enzyme conc. I.U./ gm dry wt.	Final pH	dry wt mgm /100 ml medium	enzyme conc. I.U./ gm dry wt.	Final pH
48	050	4.5	7.0	75	16.66	7.0	090	67.5	7.0	225	41.66	7.0
96	27	5.83	7.5	247	12.58	7.6	220	43.33	7.5	615	6.33	7.4
120	150	17.5	7.9	180	9.16	7.9	240	21.16	7.7	517	4.16	7.7
144	220	17.5	8.1	247	4.16	8.0	270	5.00	8.0	498	4.16	8.0
192	310	11.66	8.5	330	4.16	8.5	190	2.5	8.3	187	4.1	8.6

L-asparaginase productivity of S. karnatakensis and S. venezuelae in static and shaken cultures at different culture ages. Medium was ISA liquid medium, incubation was at 28°C for the specified period of time in static or shaken culture as indicated. Enzyme assay was carried out as described in the text.

Figure 1.
Effect of L-asparagine concentration in the growth medium on L-asparaginase productivity of S. karnatakensis and S.venezuelae. Medium was ISA liquid medium with L-asparagine conc. as specified on the figure, incubation was at 28°C for 7 days in static cultures. Enzyme assay was carried out as described in the text.

Figure 2.
Effect of the medium initial pH on L-asparaginase productivity of S. venezuelae. Medium was ISA liquid medium with its pH adjusted to the values indicated in the tables. Incubation for 7 days at 28°C in static cultures. Enzyme assay was carried out as described in the text.

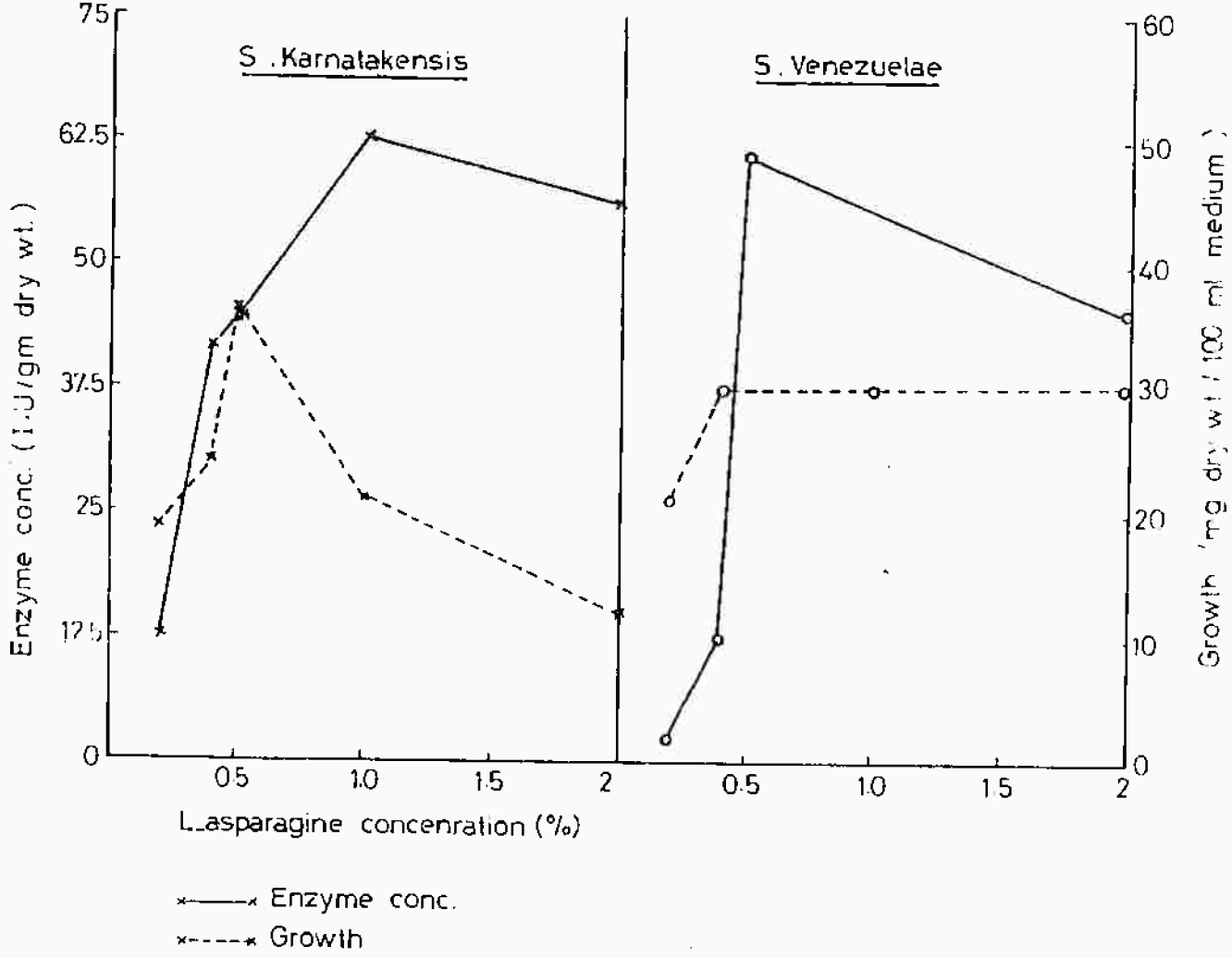
S. Karnatakensis
S. Venezuelae
Enzyme conc. (I.U/gm dry wt.)
Growth (mg dry wt / 100 ml medium)
L-asparagine concenration (%)
75
62.5
50
37.5
25
12.5
0
60
50
40
30
20
10
0
0.5
1.0
1.5
2
Enzyme conc.
Growth

Fig 1

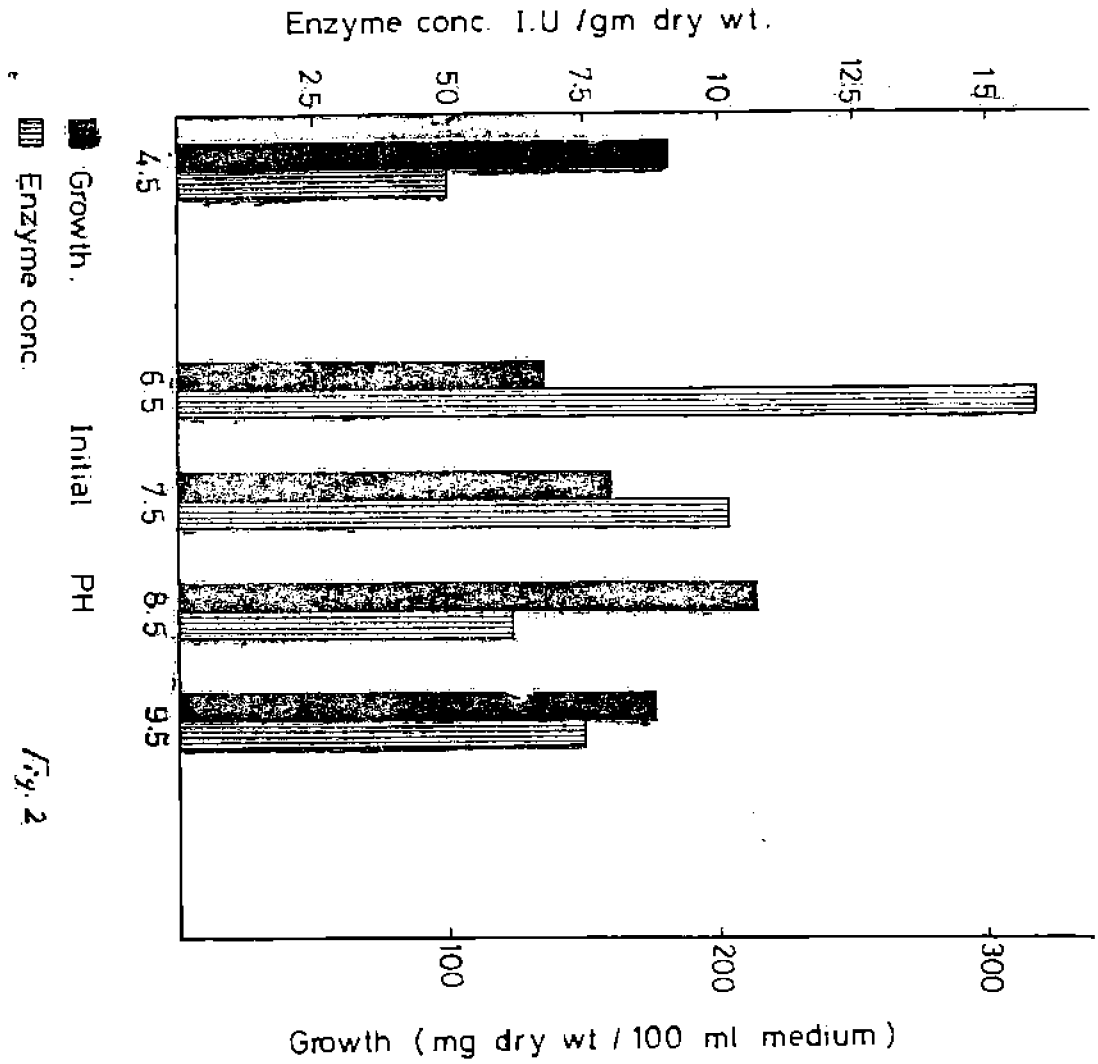
Enzyme conc. I.U /gm dry wt.
2.5
5.0
7.5
10
12.5
15
4.5
6.5
7.5
8.5
9.5
Initial PH
Growth
Enzyme conc.
100
200
300
Growth (mg dry wt / 100 ml medium)

Fig. 2

Plate 1.
Electron photomicrograph of S. venezuelae showing spore chains of the RF type and smooth spore surface.