Studies on the Amino Acids Fermentation of Pentose Materials

Part II. Formation of Amino Acids from Pentoses and Hexoses

By *Brevibacterium pentoso-aminoacidicum* nov. sp.

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Fundamental studies on the cultural conditions for the amino acids production from pentoses and hexoses employing a strain of our new isolates named *Brevibacterium pentoso-aminoacidicum* nov. sp. were carried out.

As a result of these experiments, it became possible to obtain about 30% of alanine and 10% of L-glutamic acid based on xylose, and alanine from glucose with a yield of about 40% in the proper conditions.

INTRODUCTION

In a previous paper¹⁾, we discussed the morphological and physiological properties of a new group of *Brevibacterium* isolated from soils and capable of producing amino acids from pentoses and hexoses. These new isolates were classified into two species: *Brevibacterium pentoso-aminoacidicum* and *Brevibacterium pentoso-alanicum*. *Brev. pentoso-aminoacidicum* was found to produce remarkable amounts of L-glutamic acid and alanine from pentoses and hexoses. *Brev. pentoso-alanicum* was shown to be able to convert these sugars into alanine with a high yield.

In both cases, ammonium salts were adopted as a nitrogen source.

The present paper concerns the fermentative production of L-glutamic acid and alanine from pentoses and hexoses by *Brev. pentoso-aminoacidicum*. The fundamental studies were carried out with the intension of finding out the adequate conditions for the fermentative production of these amino acids.

EXPERIMENTAL METHODS

Microorganisms. *Brevibacterium pentoso-aminoacidicum* nov. sp. was used throughout the experiments.

Analytical Methods. For the qualitative analysis of amino acids, the method of ascending paper chromatography using a mixture of *n*-butanol, acetic acid and water (4:1:4 by vol.) as a solvent followed by the detection of spots by the ninhydrin solution was adopted.

Quantitative analysis of L-glutamic acid was accomplished by the microbioassay method using *Lactobacillus arabinosus* 17-5²⁾ and the manometric method with the L-glutamic decarboxylase preparation obtained from the culture of *Escherichia coli* Crookes³⁾.

Alanine was determined by the microbioassay method using *Leuconostoc citrovorum* 8081²⁾.

Xylose and glucose were analysed by Somogyi-Shaffer-Hartman's method⁴⁾.

The extent of cell growth was expressed by the optical density at 562 m μ of the culture broth diluted to one twenty sixth with water.

Organic acids were separated by the method of paper chromatography with the use of a mixture of *n*-butanol, formic acid and water (4:1:1.5 by vol.)

2) T. Tsunoda, *Protein Chemistry*, **1**, 282 (1954).

3) W. Umbreit, R. Burris and J. Stauffer, "Manometric Techniques", 1957, p. 207.

4) P. Schaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).

1) K. Yamada and Y. Hirose, *This Journal*, **24**, 621 (1960).

as a solvent and the spots were detected with a brom-phenol blue solution. The detection of α -ketoglutaric acid and pyruvic acid was performed by the paper chromatography of their hydrazones with *n*-butanol saturated with water.

Methods of Fermentation Tests. Unless otherwise stated, the fermentation tests were conducted in the following way: Cultures were performed with 20 ml of the medium in 500 ml flasks and incubated at 30°C on a reciprocal shaker at a rate of 144 r.p.m. After 120 hours' incubation, the cells were filtered off and the filtrates were subjected to analysis.

EXPERIMENTAL RESULTS AND DISCUSSION

(I) The Influence of Nitrogen Sources.

With the intension of finding out the proper nitrogen sources for the production of amino acids, several compounds containing nitrogen were examined. The compounds examined in these experiments are the following twelve substances: NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, $\text{NH}_4\text{H}_2\text{PO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, ammonium tartarate, ammonium acetate, ammonium citrate, KNO_3 , NaNO_3 , NaNO_2 , NH_4NO_3 and urea.

For these experiments, the basal medium was composed of 5.0 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05 g of peptone in 100 ml of tap water. The pH was adjusted to 7.0. The results are shown in Table I.

No growth was observed when NaNO_2 ,

ammonium tartarate, ammonium acetate and ammonium citrate were used. And it was found that this strain has rather low assimilability of nitrates. Urea as well as the ammonium salts of the inorganic acids, $(\text{NH}_4)_2\text{SO}_4$ and NH_4Cl , were proved to be good nitrogen sources. Accordingly, urea was adopted for the nitrogen sources in the following experiments.

(II) The Influence of Urea Concentration.

The concentration of the nitrogen sources was supposed to be important for amino acids production and the effect of the concentration of urea was examined. The results were as follows (Fig. 1):

The basal medium employed was the same as before. As shown in the figure, the yield of alanine was much influenced by the nitro-

FIG. 1. Influence of Urea Concentration on the Production of Amino Acids.

TABLE I. INFLUENCE OF NITROGEN SOURCES ON THE PRODUCTION OF AMINO ACIDS

N-Sources	N-Sources %	M.W.	N—%	Yields based on the sugar added	
				Alanine %	Glutamic acid %
NH_4Cl	1.5	53.5	0.38	14.6	5.2
NH_4NO_3	1.1	80.1	0.38	5.2	2.1
$(\text{NH}_4)_2\text{SO}_4$	1.8	132.1	0.38	10.8	5.2
KNO_3	2.7	101.1	0.38	3.1	0
NaNO_3	2.3	85.0	0.38	2.8	0
NaNO_2	1.8	69.0	0.38	0	0
$\text{NH}_4\text{H}_2\text{PO}_4$	3.0	115.0	0.38	10.2	5.3
$(\text{NH}_4)_2\text{HPO}_4$	1.8	132.1	0.38	8.1	4.0
NH_4 -tart.	2.5	184.0	0.38	0	0
NH_4 -acet.	2.0	77.1	0.38	0	0
NH_4 -citr.	2.2	243.2	0.38	0	0
Urea	0.8	60.1	0.38	24.7	8.1

gen concentration but the yield of L-glutamic acid only slightly. The optimal concentration of urea for alanine production was observed to be 0.6~1.0% (corresponding to 0.3~0.5% N). Addition of too much urea caused the remarkable decrease of alanine formation and it was supposed that this was the result of the unfavorable change of pH of the culture broth owing to the strong urease activity of this strain. In the following experiments, therefore, the initial concentration of urea was fixed at 0.6~0.8% and in the course of the fermentation the feeding of this nitrogen compound was performed to maintain the moderate pH value and also to supply the nitrogen.

The optimal concentration of urea, when the feeding method was not employed, was about 0.8% (corresponding to 0.4% N), and carbon-nitrogen ratio of this medium was about 100 : 25.

(III) The Influence of Growth Substances.

It was supposed that the growth substances requirement of this strain was rather low and the addition of the slight amounts of these substances was effective for the amino acids production as well as for the growth. To ascertain this, the effects of five common organic substances, peptone, yeast extract, meat extract, corn-steep liquor and casate, were examined.

In this experiment, the basal medium was composed of 5.0 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of $MgSO_4 \cdot 7H_2O$ and 0.6 g of urea in 100 ml of tap water and to this basal medium these substances were added at three levels of concentration. The results summarized in Table II show that peptone and casate are proper growth substances for amino acids production. It is also evident that addition of too much of these compounds caused the decrease of the amino acids formation. And it is interesting that in some cases no glutamic

TABLE II. INFLUENCE OF GROWTH SUBSTANCES ON THE PRODUCTION OF AMINO ACIDS

Substances added	%	Yields based on the sugar added	
		Alanine %	Glutamic acid %
Peptone	0.05	24.7	8.1
	0.1	16.2	10.2
	0.2	8.0	8.5
Yeast extract	0.05	13.3	0
	0.1	8.5	0
	0.2	7.3	2.2
Meat extract	0.05	10.2	2.0
	0.1	9.1	2.2
	0.2	7.1	2.3
C.S.L.	0.05	10.7	0
	0.1	8.7	0
	0.2	8.0	0
Casate	0.05	13.1	0
	0.1	14.7	0
	0.2	20.0	0
Control	—	3.2	0

acid was produced even though alanine accumulated well.

More detailed experiments were carried out concerning the influence of the concentration of peptone and casate found to be adequate for amino acids production. In this experiment, the basal medium was as follows: glucose 10 g, K_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g and urea 0.6 g in 100 ml of tap water. The results obtained are as follows (Table III):

TABLE III. INFLUENCE OF PEPTONE AND CASATE ON THE PRODUCTION OF AMINO ACID

Peptone added		0%	0.05%	0.10%	0.20%
Casate added	%				
0	0	4.7*	49.8	10.7	3.5
0.01	0.01	10.0	52.7	22.0	4.0
0.02	0.02	12.1	49.7	23.4	3.7
0.04	0.04	25.8	47.5	7.3	4.1
0.06	0.06	23.1	23.6	6.5	3.7
0.10	0.10	5.1	8.0	5.8	4.0
0.20	0.20	2.6	2.5	—	3.5

* The figures indicate the yield of alanine based on glucose added.

It was observed that the optimal concentration of peptone seemed to be 0.05% and the optimal concentration of casate was likely to lie in the range of 0.01~0.04%, and peptone

was much more effective than casate only. This fact means that the effective substances are included in peptone rather than in casate, and as the casate used here was the vitamin-free preparation, this effective substances are supposed to be a kind of vitamins.

(IV) The Vitamin Requirements for Growth.

In the previous section, it was supposed that some of the vitamins were effective for amino acids production, and in this connection the vitamin requirements of this strain for growth were examined. The minimal medium was composed of 2% of xylose, 0.1% of K_2HPO_4 , 0.05% of $MgSO_4 \cdot 7H_2O$ and 0.6% of urea. Vitamin-free casamino acids and several vitamins were added to this minimal medium and the growth was determined after 18 hours' cultivation. The results are shown in Table IV. The seed culture employed in this experiment was the washed cell suspension harvested from bouillon culture.

In the absence of biotin, the growth hardly occurred and it might suggest the requirement for biotin in the growth of this strain. As for

TABLE IV. VITAMINE REQUIREMENTS FOR GROWTH

Medium Composition	Growth (O.D.)
M.M.	0.010
M.M.+A.A.	0.040
M.M.+A.A.+V.	0.213
// —Biotin	0.060
// —Thiamine	0.098
// —Pantothenic acid	0.148
// —Nicotinic acid	0.218
// —Riboflavin	0.218
// —Folic acid	0.188
// — <i>p</i> -Aminobenzoic acid	0.208
// —Choline	0.203
// —Inositol	0.210
// —Pyridoxine	0.163

M.M.: Minimal medium.

A.A.: Vitamine-free casamino acids.

V.: All the vitamins mentioned below:

Biotin: 0.2 γ /dl, Thiamine: 10 γ /dl (hydroxide), Pantothenic acid: 10 γ /dl (Ca salt), Nicotinic acid: 50 γ /dl, Riboflavin: 20 γ /dl, Folic acid: 10 γ /dl, *p*-Aminobenzoic acid: 10 γ /dl, Choline: 500 γ /dl (chloride), Inositol: 50 γ /dl, Pyridoxine: 10 γ /dl (hydrochloride).

the other vitamins, the results were not obvious.

(V) The Influence of Carbohydrates Concentration.

Xylose and glucose were employed as carbon sources and the influence of their concentration on the amino acids production was examined. The basal medium in this experiment was composed of 0.1 g of K_2HPO_4 , 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of the peptone and 0.6 g of urea in 100 ml of tap water. The results are shown in Fig. 2 and 3.

The tendencies were the same in both substrates, and it was found that this strain showed greater resistance to glucose than to xylose. And it was also observed that the optimal initial concentration of xylose and glucose seemed to be about 5% and 10%, respectively. It seemed to be the reason why the yields of amino acids were lower when the sugar concentrations were below or above

FIG. 2. Influence of Xylose Concentration on the Production of Amino Acids.

FIG. 3. Influence of Glucose Concentration on the Production of Amino Acids.

these optimal values that the concentration of peptone was not adequate in these cases. Accordingly it should be possible to maintain higher yields of amino acids by adjusting the concentration of peptone according to the concentration of sugar.

(VI) The Influence of Bulk Salts.

As the requirements of this strain for the organic growth substances were rather low, it was supposed that the effects of inorganic salts on the amino acids production should be important, and in the following experiments the influence of bulk salts such as K_2HPO_4 , KH_2PO_4 , $MgSO_4 \cdot 7H_2O$ and NaCl was examined.

(1) Effects of K_2HPO_4 .

In this experiment, the basal medium was as follows: xylose 5 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, peptone 0.05 g and urea 0.6 g in 100 ml of tap water. The results are shown in Fig. 4.

FIG. 4. Influence of K_2HPO_4 Concentration on the Production of Amino acids.

L-Glutamic acid production was rather indifferent but alanine productivity was much influenced by the concentration of K_2HPO_4 and the optimal concentration of this salts was found to be about 0.1%. An excess of K_2HPO_4 resulted in the decreased yield of alanine.

(2) Effects of KH_2PO_4 .

In this experiment, the basal medium was composed of 5 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of peptone and 0.6 g of urea in 100 ml of tap water. As shown

FIG. 5. Influence of KH_2PO_4 on the Production of Amino Acids.

in Fig. 5, the optimal concentration of KH_2PO_4 lies between 0.07 and 0.13% and in this case, too, an excess of it had bad effects on alanine production.

(3) Effects of $MgSO_4 \cdot 7H_2O$.

In this experiment, the basal medium composition was 5 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of peptone and 0.6 g of urea in 100 ml of tap water. The results are as follows (Fig. 6):

FIG. 6. Influence of $MgSO_4 \cdot 7H_2O$ Concentration on the Production of Amino Acids.

These show that more than 0.1% of $MgSO_4 \cdot 7H_2O$ had a favorable effect on the alanine accumulation, but as the concentration of this salts increased, the productivity of L-glutamic acid seemed to decrease.

(4) Effects of NaCl.

In this experiments, the basal medium was

FIG. 7. Influence of NaCl Concentration on the Production of Amino Acids.

as follows: xylose 5 g, K_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, peptone 0.05 g and urea 0.6 g in 100 ml of tap water.

No stimulatory effect was observed as shown in Fig. 7.

(VII) The Influence of Trace Elements.

The effects of eight metals on the amino acids production were examined. Basal medium employed was as follows: xylose 5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, peptone 0.05% and urea 0.6%.

The results appear in Table V. It was observed that Mn^{++} gave a marked stimulation but Ni^{++} , Cu^{++} , Zn^{++} and Cd^{++} had poisonous effects. Fe^{+++} , Mo^{++} and Co^{++} had no noticeable influences.

(VIII) The Influence of Oxygen Supply.

In order to investigate the influence of oxygen supply on the amino acids formation,

the following experiments were carried out. The medium employed was composed of 5 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of peptone and 0.6 g of urea in 100 ml of tap water. Incubation was performed with several volumes of this medium in 500 ml flasks using a reciprocal shaker (70 mm stroke) at a rate of 144 r.p.m. at 30°C. The extent of oxygen supply was expressed by the oxygen adsorption coefficients, K_d [g mol of O_2 /ml min atm] determined by the sulfite methods⁵⁾.

The results are shown in Fig. 8.

FIG. 8. Influence of Oxygen Supply on the Production of Amino Acids.

It was obvious that the aerobic condition was preferable for this fermentation but too high oxygen supply was not adequate. The recommended rate of oxygen supply was performed when about 40 ml of the medium was

TABLE V. INFLUENCE OF TRACE ELEMENTS ON THE PRODUCTION OF AMINO ACIDS

Elements	Compound added		Growth	Yields based on the sugar added	
				Alanine %	Glutamic acid %
Fe^{+++}	$Fe_2(SO_4)_3$	10 ppm	+	7.8	14.3
Mn^{++}	$MnSO_4 \cdot 7H_2O$	//	+	20.5	12.4
Mo^{6+}	Na_2MoO_4	//	+	11.7	12.6
Co^{++}	$CoSO_4 \cdot 7H_2O$	//	+	9.8	4.9
Ni^{++}	$NiSO_4 \cdot 7H_2O$	//	+	0	0
Cu^{++}	$CuSO_4 \cdot 5H_2O$	//	+	0	0
Zn^{++}	$ZnSO_4 \cdot 7H_2O$	//	+	0	0
Cd^{++}	$CdSO_4 \cdot 7H_2O$	//	-	0	0
Control	—	—	+	9.3	14.4

5) K. Yamada, J. Takahashi and H. Okada, *J. Agr. Chem. Soc. Japan*, **27**, 704 (1953).

employed in 500 ml flask under the condition mentioned above. This recommended rate corresponds to $9\sim 10\times 10^{-6}$ Kd.

(IX) Course of the Fermentation.

An example of the time-course studies of this fermentation is shown in Fig. 9. The medium used was composed of 5 g of xylose, 0.1 g of K_2HPO_4 , 0.05 g of $MgSO_4\cdot 7H_2O$, 0.05 g of peptone and 0.6 g of urea in 100 ml of tap water.

FIG. 9. An Example of this Amino Acids Fermentation.

●—●—● xylose
 △—△—△ alanine
 ---○--- growth
 x—x—x glutamic acid

By-products such as acetic acid and pyruvic acid were detected in the culture broth. Alanine produced by this fermentation was identified to be a racemic form by the infra-red analysis and also by the biological method using D-amino acids oxidase.

SUMMARY

In order to find out the proper conditions for the fermentative production of the amino acids by *Brevibacterium pentoso-aminoacidicum* nov. sp., the effects of several factors were studied.

Nitrogen sources such as NH_4Cl , $(NH_4)_2SO_4$ and urea were examined as to their adequacy for the production of amino acids by this strain and urea was found to be the best of all compounds tested. The proper range of the concentration of this nitrogen source was determined to be 0.6 to 0.8%.

As for the growth substances, peptone and casate were shown to have excellent effects in the concentration of about 0.05% and it was also found that this strain required biotin for its growth.

The proper concentration of carbon sources, xylose and glucose, were found to be about 5% and 10%, respectively.

The effects of several minerals including bulk salts and trace elements were also discussed. The adequate concentration of K_2HPO_4 , KH_2PO_4 and $MgSO_4\cdot 7H_2O$ were about 0.1%, and Mn^{++} showed a marked stimulating effect on the amino acids production.

The aerobic condition was favorable for growth and amino acids production and the desirable degree of oxygen supply was determined to be about $9\sim 10\times 10^{-6}$ Kd [g mol of O_2 /ml min atm].

Alanine produced by this fermentation was found to be a racemic form.