Studies on the decarboxylation of amino acids by *Brevibacterium linens**

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During the course of investigation of the characteristic flavour produced by *Brevibacterium linens*, amines were found to be major components of the flavour¹⁶⁾. From the quantitative estimation of amines produced in Medium-S (glucose 1 %, meat extract 1 %, peptone 1 % and NaCl 0.5 %) cultivated with *Brev. linens*, monomethyl amine, monoethyl amine, triethyl amine, diethyl amine, cadaverine, tyramine and histamine were detected¹⁵⁾. Amongst these, cadaverine was found to be significantly produced by this organism¹⁵⁾.

Although the production of amines as a result of bacterial putrefaction has been recognized since the beginning of the present century¹¹⁾, and extensive studies on the production of amines by bacterial amino acid decarboxylase have been made by GALE^{3,4,5,6)} and other workers^{8,18)}, we have yet very little informations as regards the production of amines by amino acid decarboxylases of many cheese starter organisms including *Brev. linens*.

In the present report, the decarboxylation of amino acids by *Brev. linens* was manometrically investigated, together with the qualitative estimation of amines produced by the amino acid decarboxylase of this strain.

Experimental

1. Strain used: *Brev. linens* which was previously described^{14,16)} was used for the experiments. This strain was incubated at 21°C and transferred daily to Medium-S.

2. Methods for estimating the effects of additives to rennet whey on volatile amine formation: Acid hydrolyzed casein (casamino acids, Difco) and L-lysine were used as additives to rennet whey medium. Rennet whey, the one containing 0.50 or 0.25 % acid hydrolyzed casein and the other containing 2 % of 0.05 M lysine solution were prepared.

One hundred ml of each of these media was adjusted to pH 7.0 and dispensed in a culture flask (250 ml capacity). After sterilization at 120°C for 10 min., 20 ml of 48 hrs' culture of *Brev. linens* in Medium-S was inoculated into each of these media and they were incubated at 21°C for 48 hrs with reciprocal agitation (140 reciprocation per minute).

Total amounts of bases produced in these media were estimated by the same method as described in previous papers^{7,16)}.

3. General methods for preparation and activity measurement of the amino acid decarboxylase: Acetone powder of *Brev. linens* cells was prepared from 72 hrs' culture.

Three hundred mg of the powder was suspended in 15 ml of M/45 borate buffer at pH 8.0.

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The suspension was incubated for 2 hrs at 30°C and centrifuged. The sediment then was spun off and the clear supernatant was used as the crude amino acid decarboxylase of *Brev. linens*.

The decarboxylation of amino acids was followed by measuring the evolution of CO₂ in a Warburg manometric apparatus (Koshin-Kagakukiki-Seisakusho, E 13 Type). The main cup of the Warburg manometer contained 2 ml of M/5 phosphate buffer and 0.5 ml of the enzyme preparation, with 0.5 ml of M/15 single amino acid solution in the side-bulb. The manometers were equilibrated in a thermostat and the contents of the side cup tipped into the main compartment after equilibration.

The CO₂ evolution was measured for 1 hr after tipping. The following L-amino acids were used for the experiment; glycine, alanine, valine, leucine, threonine, glutamic acid, aspartic acid, lysine, proline, arginine, histidine, tyrosine and tryptophan.

4. Identification of amines produced from amino acids:

Single amino acid (alanine, leucine, glutamic acid, lysine, tyrosine and histidine) was resolved in M/5 phosphate buffer at pH 7.0 so as to contain 0.05 M. One ml of resting cells of *Brev. linens* prepared by the same method as described in the previous paper⁷⁾ (about 100 mg dry weight of cells per ml.) was added to 100 ml of each of the amino acid solution and incubated at 30°C for 24 hrs with shaking. Produced amines were qualitatively identified by paperchromatography in the same manner as described in the previous papers^{15,16)}, using solvent system of n-butanol-acetic acid-water (4 : 1 : 5 in volume).

Results and Discussion

1. Effect of the addition of acid hydrolyzed casein and lysine to rennet whey

In the present experiments, at first, effects of the addition of acid hydrolyzed casein and lysine to rennet whey media were investigated.

The amounts of total bases (ml of 0.1 N HCl/50 ml of medium) in these media before and after incubation are shown in Table 1, which clearly show that amounts of total volatile bases after incubation generally increased in the medium containing acid hydrolyzed casein or lysine compared to the plain rennet whey medium. Especially, the production of volatile base is the most significant in the rennet whey medium containing 0.5 % acid hydrolyzed casein.

These results apparently indicate that a part of amino acids is easily converted to volatile amines by *Brev. linens*.

Table 1. Effect of the addition of acid hydrolyzed casein and lysine to rennet whey medium on the formation of volatile bases

Medium	Titrateable bases of steam-distillate (0.1 N HCl/50 ml of medium)
Before incubation, whey	2.0
After incubation, whey	6.1
whey+0.25% acid hydrolyzed casein	10.5
whey+0.5% acid hydrolyzed casein	17.8
whey+2 ml of 0.05 M lysine solution	8.7

Incubation; 48 hrs at 21°C

2. Effect of pH of substrate on the decarboxylation of amino acids

In order to investigate this problem, manometers were set up containing similar amounts

Fig. 1. Effect of pH on the decarboxylation of amino acids at 30°C

of organism, etc., in the presence of buffers at pH ranging from 4.0 to 9.0 with intervals of 0.5 pH unit. The resulting activities were expressed as Q_{CO_2} ($= \mu l$ evolved CO_2 /mg dry weight of bacteria/hr). As shown in Fig. 1, apparent evolution of CO_2 was obtained with lysine, tyrosine, glutamic acid, alanine, leucine, histidine and glycine. These amino acids except glycine and histidine were decarboxylated optimally at pH 7.0, and the evolution of CO_2 from either of these amino acids decreased more rapidly when the reaction occurred at alkaline side than when the pH was lowered.

Special meaning of this phenomenon is that most of the amino acids were decarboxylated optimally at neutral pH. This phenomenon is strictly different from the cases of the bacterial decarboxylases described by GALE^{3,4,5,6)} and other workers^{1,12)}, who reported that decarboxylations occur optimally at acidic pH range from 4.0 to 5.0.

Although the decarboxylases which have their optimal activities at neutral pH were also found in a contain bacteria¹⁶⁾ or in the stomach of rat¹⁰⁾, the results obtained from the present study, especially, are considered to have an important relation to the development of characteristic flavour in Limburger cheese, because the pH at the surface of Limburger cheese ripened for two months was known to be usually 6.20~6.80³⁾.

As also seen from Fig. 1, decarboxylation of lysine is the most remarkable among these amino acids, and lysine has Q_{CO_2} of the order 8.0~11.5 between pH 5.0~7.0. In our previous paper¹⁶⁾, it was shown that *Brev. linens* produced cadaverine significantly. Therefore, the present results are a possible explanation to account for the previous results.

3. Effect of temperature on decarboxylation of amino acids.

Since significant decarboxylations were generally observed with lysine, alanine, leucine, glutamic acid and tyrosine at each pH value between 5.0~7.0 and 30°C, the decarboxylation was investigated at various temperature using these amino acids. Vessels containing the usual quantities at the optimum pH of 7.0 were set up in thermostats at 20, 25, 30, 35, 40, 45 and 50°C, respectively.

Q_{CO_2} values after 1 hr reaction at the various temperature are shown in Fig. 2, from which it is noted that the variation of the activities with the reaction temperature is different

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Fig. 2. Effect of temperature on the decarboxylation of amino acids at pH 7.0

Fig. 3. Changes of the amino acid decarboxylases activities with age of the culture

among these amino acid decarboxylases. The optimum temperature for decarboxylation of these amino acids is 30°C for leucine and tyrosine, and 35°C for lysine, alanine and glutamic acid, respectively. Furthermore, reaction with glutamic acid, leucine or tyrosine is much sensitive to temperature, that is, no CO₂ was liberated from these amino acids at 20°C.

4. Effect of age of culture on the activities of amino acid decarboxylase

To investigate the variation of activity of the amino acid decarboxylase with the age of incubation, crude amino acid decarboxylases were prepared from 0, 24, 48, 72 and 96 hrs' cultures of *Brev. linens* which grew in Medium-S (initial pH 6.0). Changes of pH of the medium during incubation were also estimated.

The activities of these decarboxylases toward lysine, alanine, leucine, glutamic acid and tyrosine were determined separately under optimal conditions and the results are shown in Fig. 3.

As readily be seen in Fig. 3, early cultures have generally little activity, the activity increasing rapidly between 24 to 48 hrs of growth, becoming maximal at 48 to 72 hrs. pH of

the culture also rose with the increase of incubation period and reached 7.0 and 8.0 after 48 and 72 hrs, respectively.

GALE et al.⁶⁾ reported that when bacteria are grown in an alkaline medium, they attack amino acids by deamination, and when growth occurs in an acid medium, by decarboxylation, and that it is more satisfactory for producing amino acid decarboxylase to cultivate the organism in a medium containing glucose or other fermentable carbohydrate; the fermentation of the carbohydrate during growth greatly increases the cells and, at the same time, causes a fall of pH.

However, in the present study apparent decarboxylation occurred in the cultures of which pH are within alkaline range. When *Brev. linens* was cultivated in a glucose containing medium (Medium-S), pH of the medium was rather risen from 6.0 to 8.3, although the enzymatic activity was enhanced by the addition of glucose to the medium (Table 2). The facts described above definitely indicate that glucose is certainly satisfactory to enhance the activity of the decarboxylase but does not reduce pH of the culture of *Brev. linens*.

Table 2. Effect of addition of glucose to the medium on the production of amino acid decarboxylases by *Brev. linens**

	lysine	glutamic acid	leucine	alanine	tyrosine
	(Qco ₂)				
2% glucose containing medium**	11.4	5.7	5.9	5.2	3.5
glucose free medium**	8.5	3.4	4.3	3.8	2.2

* Crude amino acid decarboxylase was prepared from the 48 hrs' culture and the activities of the decarboxylase toward lysine, glutamic acid, leucine, alanine and tyrosine were determined under optimal conditions.

** Basal medium is composed of meat extract 1%, peptone 1%, and NaCl 0.5%.

5. Identification of produced amines

Amines produced from lysine, alanine, leucine, glutamic acid and tyrosine were qualitatively investigated by paperchromatography.

As the results, cadaverine (Rf=0.17, purple color in ninhydrin reaction), monomethyl amine (Rf=0.24, purple color in ninhydrin reaction), isoamylamine (Rf=0.69, purple color in ninhydrin reaction), γ -amino butyric acid (Rf=0.40, purple color in ninhydrin reaction) and tyramine (Rf=0.52, orange color in diazo reaction) were produced from lysine, alanine, leucine, glutamic acid and tyrosine, respectively.

Summary

The decarboxylation of amino acids by *Brevibacterium linens* was manometrically investigated.

From the fact that the formation of volatile amines in amino acid rich media was significantly higher, it was considered that amino acids were easily decarboxylated by *Brev. linens*. The crude amino acid decarboxylase of *Brev. linens* was then extracted from the acetone-dried organism, and the enzymatic activities toward thirteen amino acids such as glycine etc. were determined under various conditions. The results obtained were as follows:

(1) Significant decarboxylations were generally observed with lysine, alanine, leucine, glutamic acid and tyrosine at pH value between 5.0 to 7.0 and 30°C. Leucine and tyrosine were decarboxylated optimally at pH 7.0 and 30°C, and lysine, alanine and glutamic acid were at pH 7.0 and 35°C.

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Decarboxylation of lysine was the most remarkable among these amino acids, and lysine had Q_{CO_2} of the 13.0 at pH 7.0 and 35°C.

(2) Activities of the amino acid decarboxylase were varied with the age of the culture; pH of the culture gradually rose to alkaline pH with the increase of incubation period and the decarboxylases prepared from the 48 and the 72 hrs' cultures had maximal activity toward alanine, and lysine, leucine, glutamic acid and tyrosine, respectively.

These enzymatic activities were enhanced by addition of glucose to the culture medium but glucose did not reduce pH of the culture of *Brev. linens*.

(3) Amines produced from lysine, alanine, leucine, glutamic acid and tyrosine were cadaverine, monomethyl amine, isoamyl amine, γ -amino butyric acid and tyramine, respectively.

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Brevibacterium linens によるアミノ酸の脱炭酸に関する研究

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レンネットホエー培地にカゼイン酸加水分解物またはリジンを添加すると揮発性塩基の生成が顕著に増加することから、アミノ酸が *Brevibacterium linens* により容易に脱炭酸されることが予想された。そこで *Brev. linens* のアセトン粉末菌体より抽出した粗アミノ酸デカルボキシラーゼをグリシンなど 13 種の L-アミノ酸に種々の条件で作用させ、遊離する CO_2 をワールブルグ検圧計により測定した。得られた結果は次のとおりである。(1) リジン、アラニン、グルタミン酸およびチロシンは基質 pH が 5.0 から 7.0 の範囲で明らかに脱炭酸されるのが認められ、それらアミノ酸が脱炭酸される至適条件はロイシンとチロシンが pH 7.0, 30°C およびリジン、アラニン、グルタミン酸が pH 7.0, 35°C であった。中でもリジンはいずれの条件下においてもきわめて顕著に脱炭

酸され、リジンデカルボキシラーゼの活性が特異的に強いことを認めた。(2) *Brev. linens* を培養すると培地の pH が次第にアルカリ側に移行し、アラニンデカルボキシラーゼは培養 48 時間目の培地から調製したものにおいてその活性が最高であり、またリジン、ロイシン、グルタミン酸、チロシンのそれぞれのデカルボキシラーゼは培養 72 時間目の培地から調製したものにおいていずれも活性が最大であった。また、培地にグルコースを添加すると産出されるデカルボキシラーゼの活性は高まるが、グルコース添加によって培養液の pH は酸性側に移行せず GALE らの見解と異なった結果を示した。(3) リジン、アラニン、ロイシン、グルタミン酸、チロシンからはそれぞれカダベリン、モノメチルアミン、*i*-アミルアミン、 γ -アミノ酪酸およびチラミンが生成された。