tion of levulinic acid oxime did not influence the urinary excretion of sodium and potassium in normal rats.

The sodium salt of levulinic acid oxime was injected into mice in order to measure the remaining cesium-134 in mice organs. The decrease of cesium-134 retention was observed in liver, but not in muscle, testis, spleen, and the kidneys. This point would be noteworthy in our future study.

The two oximes examined in this paper were almost non-toxic. LD₅₀ of the oximes were

2040 mg/kg (levulinic acid oxime, mice, intravenously), and 3500 mg/kg (α -ketoglutaric acid oxime, intraperitoneally), respectively.

Acknowledgements The author wishes to express his sincerest thanks to Prof. Y. Sumiki for his encouragement and guidance through these experiments. He is thankful to Mr. Ko-ling Yeh and Miss H. Toyozumi, Department of Agricultural Chemistry, University of Tokyo, for their contributions in the course of this experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 5, p. 489~496, 1960]

Betaine as a Growth Factor for *Pediococcus soyae*

Part VIII. Studies on the Activities of Bacteria in Soy Sauce Brewing

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Received December 9, 1959

Besides P-factor¹⁾, *Pediococcus soyae*^{2,3)} requires betaine (glycine-betaine) as a specific growth promotant. The maximal growth is obtained with the supplementation of both betaine and P-factor to the synthetic medium, while betaine only gives the half-maximal growth. When the organism is cultured in 18% salted media, the addition of both betaine and P-factor is essential for the occurrence of growth. Thus, betaine is requisite for *Ped. soyae* as a growth promoting factor and also as a factor bestowing the osmotolerant ability.

Pediococcus soyae is characterized by three eminent specificities, namely, by its acid-sensitivity²⁾, by its osmotolerant character to grow even in 24~26% salted media^{1,2)}, and by its unique nutritional requirements¹⁾. This organism does not grow in the ordinary synthetic medium for lactic acid bacteria, and requires

two additional growth factors. One of them, which was provisionally named as P-factor in the preceding papers, is found to be included in partial hydrolysates of Hammersten's milk casein prepared with hydrochloric acid or various crystalline proteinases. The other, S-factor, is found to be present in Difco's yeast extract, beef extract, heart infusion broth, in soy sauce, and in the water extract of *Aspergillus soyae* mycelium. The maximal growth is, however,

1) Kenji Sakaguchi, This Bulletin, **23**, 438, 443 (1959).

2) Kenji Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22, 100 (1959); *Rep. Noda Inst. Sci. Res.*, **2**, 40 (1958).

3) K. Yamazato and H. Iizuka, *J. Agr. Chem. Soc. Japan*, **33**, 379, 383 (1958); *J. Gen. Appl. Microbiol.*, **5**, 58 (1959).

obtained by the addition of both of the two factors; P-factor alone, giving rise to only the half-maximal growth.

This report deals with the betaine requirement of *Ped. soyae* and the fact that the S-factor and

betaine are probably identical. Some results obtained from the examination of specific nutritional requirements of the organism under salt-tolerant conditions are also described.

METHODS

I. Assay procedure.

The basal synthetic medium employed was essentially the same as that described by Snell and others⁴⁾ with the following alteration. The supplementation with choline 2 mg/l, inositol 2 mg/l and leucovorin 10 γ /l was made. Pyridoxal was omitted because it is replaceable with pyridoxine. L-Leucine and L-methionine were each substituted by two-fold amounts of DL-leucine and DL-methionine.

Stock cultures of *Ped. soyae* strain d 2 were maintained on 7% salted yeast extract-peptone-glucose-agar slabs and transferred monthly. Inocula were grown in the following medium; yeast extract (Difco) 0.3%, Polypeptone (Takeda) 1.0%, glucose 1.0%, sodium chloride 0.5%, sodium acetate trihydrate 3.3% and dipotassium phosphate 0.5%, pH 7.0. After four days of incubation at 30°C, the cells were centrifuged, washed four times with 0.9% potassium chloride solution, and diluted to give a final optical density of 0.07 at 660 m μ . Five-ml culture fluids containing appropriate supplements were prepared in the usual manner¹⁾, autoclaved for fifteen minutes at 120°C, cooled, incubated with one drop per tube of the cell suspension described above and incubated at 30°C. Growth was estimated photometrically at 660 m μ in a spectrophotometer. When the cells were cultured in 18% salted media, inocula were grown in 18% salted broth, and washed with a solution containing 18% sodium chloride, 1.0% glucose, and 3.3% sodium acetate trihydrate, pH 6.0. This solution is favourable for keeping the viability of the inocula grown in salted media.

RESULTS

I. Requirements of both P- and S-factors in salted environments.

When cultured in unsalted media, *Pediococcus soyae* shows half-maximal growth with the supplementation of P-factor alone, and maximal growth is observed by the addition of both P- and S-factors¹⁾. In 18% salted media, however, nothing was known on nutritional requirements. The results shown in Fig. 1 indicate that, under

TABLE I. COMPOSITION OF THE BASAL-SYNTHETIC MEDIUM

DL-Ala	200 mg/l
DL-Asp	400
L-Glu	500
L-Arg·HCl	200
L-Lys·HCl	200
L-His·HCl	100
DL-Ileu	200
DL-Met	200
DL-Phe	200
L-Pro	100
DL-Thr	200
L-Tyr	100
DL-Val	200
DL-Try	100
L-CyS	100
DL-Ser	100
Gly	100
L-Leu	100
Glucose	20 g/l
Na-actate·3H ₂ O	33
K ₂ HPO ₄	500 mg/l
KH ₂ PO ₄	500
MgSO ₄ ·7H ₂ O	200
FeSO ₄ ·7H ₂ O	10
MnSO ₄ ·4H ₂ O	10
NH ₄ Cl	3 g/l
Adenine sulphate	10 mg/l
Guanine HCl	10
Uracil	10
Xanthine	10
Riboflavine	2
Thiamine·HCl	1
P.A.B.A.	1
Pyridoxine·HCl	1
Ca-dl-Pantothenate	1
Nicotinic acid	1
Biotin	10 γ /l
Folic Acid	10
Choline	2 mg/l
Inositol	2
Leucovorin	10 γ /l

pH 7.0, Pyridoxal is omitted.

4) E.E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946-47); G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).

FIG. 1. Nutritional Requirements of *Ped. soyae* in Salted and Unsalted Media.

—x— Synthetic medium
—△— + Polypepton 1.0%
—○— + Polypepton 1.0% & Yeast extract 0.3% } NaCl 0.02%
---x--- Synthetic medium
---△--- + Polypepton 1.0%
---○--- + Polypepton 1.0% & Yeast extract 0.3% } NaCl 18.0%

the salted environment, no growth was observed by the mere addition of P-factor (Polypepton Takeda 1.0%) and the addition of both P- and S-factors was absolutely needed for the occurrence of growth. In the cultures with heavily salted media a longer lag period of more than six days resulted, and the final growth was also poorer.

II. Detection of the effective substance amongst various vitamins and growth stimulants.

Seventy-six vitamins and growth stimulants⁵⁻¹³⁾ were collected and their effects on *Ped. soyae* were examined. The inspection was made after four days' culture in unsalted media, and after fifteen days' culture in 18% salted media supplemented with P-factor. Amongst various growth promotants only betaine was found to be effective in both salted and unsalted media.

III. Optimum concentration of betaine hydrochloride.

To the unsalted synthetic medium, betaine

TABLE II. EFFECT OF GROWTH PROMOTANTS ON *Ped. soyae*

	μg/ml	NaCl 0.02%	NaCl 18% polypepton 1.0%
none		0.01	0.00
polypepton	10.000	0.53	0.05
polypepton + yeast-extract		0.76	0.14
polypepton + beef-extract		—	0.15
betaine HCl	30	0.45	0.00
betaine HCl + polypepton		0.67	0.17
ergosterol	10	0.00	0.04
cholesterol	//	0.00	0.02
progesterone	//	0.00	0.02
α-tocopherol	//	0.00	0.08
ATP	//	0.03	0.04
FMN	//	0.02	0.04
FAD	//	0.03	0.05
DPN ¹⁴⁾	//	0.02	0.05
TPN	//	0.03	0.05
coenzyme A	//	—	0.05
yeast RNA	//	0.03	0.05
spleen DNA	//	0.00	0.04
adenosine ¹⁵⁾	//	0.02	0.05
adenylic acid	//	0.01	0.04
deoxyadenosine	//	0.01	0.05
deoxyadenylic acid	//	0.01	0.04
cytidine	//	0.01	0.04
cytidylic acid ¹⁶⁾	//	0.03	0.04
deoxycytidine	//	0.02	0.04
deoxycytidylic acid	//	0.01	0.04
guanosine	//	0.02	0.04
guanylic acid	//	0.03	0.04
deoxyguanosine	//	0.02	0.06
deoxyguanylic acid	//	0.03	0.04
uridine	//	0.04	0.05
uridylic acid ¹⁶⁾	//	0.01	0.04
thymine	//	0.01	0.06
thymidine ^{17,18)}	//	0.03	0.05
thymidylic acid	//	0.04	0.04
Panvitan solution	1000	0.00	0.04
VB ₁₂ ¹⁹⁾	10	0.00	0.06

14) K. Kitahara and A. Ôbayashi, *J. Agr. Chem. Soc. Japan*, **33**, 497 (1959).

15) T. Fukami, Lecture given on July 4th, 1959 at the First Meeting of the Symposium on Microbioassay.

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17) E.A. Felton and C.F. Niven, *J. Bact.*, **65**, 482 (1953).

18) E. Kitay and E.E. Snell, *ibid.*, **59**, 727 (1950); **60**, 49 (1950).

19) I. Kusaka and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **33**, 703 (1959).

- 5) E.E. Snell, *Ann. Rev. Microbiol.*, **3**, 97 (1949).
- 6) W.A. Krehl and S.J. Kiao, *ibid.*, **5**, 129 (1951).
- 7) J.L. Stokes, *ibid.*, **6**, 29 (1952).
- 8) V.H. Cheldelin and T.E. King, *ibid.*, **7**, 113 (1953).
- 9) D. Hendlin, *ibid.*, **8**, 47 (1954).
- 10) E.L.R. Stockstad et al., *ibid.*, **9**, 111 (1955).
- 11) L.D. Wright, *ibid.*, **10**, 141 (1956).
- 12) B. Magasanik, *ibid.*, **11**, 221 (1957).
- 13) B.M. Guirard, *ibid.*, **12**, 247 (1958).

VK ₃	10	0.00	0.07
niacin amide	//	0.02	0.05
ascorbic acid	100	0.01	0.07
mevalonic acid (synth.) ^{20, 21)}	10	0.02	0.04
" (natural)	//	—	0.06
lipoic acid ²²⁾	//	0.01	0.05
orotic acid ^{23, 24)}	//	0.03	0.05
oleic acid ^{25, 26)}	//	0.03	0.06
DL-desthiobiotine	//	0.01	0.03
DL-pantoil lactone	//	0.03	0.05
taurine	//	0.03	0.05
tween 80 ^{25, 27)}	200	0.04	0.05
N-acetyl glucosamine ²⁸⁻³⁰⁾	100	0.01	—
β -alanine	10	0.02	0.05
D-glutamic acid ³¹⁾	100	0.03	0.04
diiodotyrosine	10	0.01	—
thyroxine	//	0.02	—
insulin	100	0.01	0.07
glutamine	//	0.01	0.04
asparagine	//	0.01	0.04
glutathione	//	0.01	0.04
Pro-Leu anhydride ³²⁾	//	—	0.03
Pro-Val anhydride ³²⁾	//	—	0.03
Pro-Leu	//	—	0.03
Pro-Val	//	—	0.03
γ -amino butyric acid	//	0.01	0.04
oxaloacetic acid	100	0.01	—
Ba-glucose-6-phosphate	//	—	0.04
Ba-hexose-1,6-diphosphate	//	0.02	0.05
Na- β -glycerophosphate	//	0.01	0.05
dihydroxyacetone	//	0.01	—
fumaric acid	//	0.00	—
lactic acid	//	0.00	—
succinic acid	//	0.00	—
glycolic acid	//	0.00	—
malic acid	//	0.00	—

citric acid	//	0.01	—
DL-glyceraldehyde	//	0.01	—
Ba-phosphoglycerate	//	0.01	—
pimelic acid	10	0.01	0.04
L,L-diamino pimelic acid ^{33, 34)}	//	0.01	0.05
L,D-diamino pimelic acid	//	0.01	0.05
D,D-diamino pimelic acid	//	0.00	0.06
rutin	//	0.03	0.05

FIG. 2. Optimal Concentration of Betaine Hydrochloride.

—○— 9 days incubated.
---x--- 10 days incubated.

hydrochloride was added in concentrations of 0.1, 1.0, 3.0, 10, 30, 100, 300, and 1000 mg/l and the growth response was measured after nine and ten days of culture. Fig. 2 shows that the best growth was obtained with the addition of 30 mg betaine hydrochloride per litre. However, even in this optimum concentration, the supplement of betaine alone resulted in the half-maximal growth. After nine days of culture, the synthetic media attained the final growth of 0.25 optical density, the supplement of 1.0% Polypepton 0.43, betaine hydrochloride 30 mg/l 0.46, while media added with Polypepton 1.0% and yeast extract 0.3% attained the optical density of 0.64. It is thus approvable that besides betaine, another growth factor is necessary for the maximal growth of *Ped. soya*.

IV Effects of various amines and related substances with betaine.

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 22) L.J. Reed et al., *Science*, **114**, 93 (1951).
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 25) Y. Tani and S. Tatsumi, *J. Ferment. Technol.*, **36**, 311, 317 (1958).
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 31) S.A. Koser and J.L. Thomas, *J. Bact.*, **73**, 477 (1957).
 32) Y. Koaze, This Bulletin, **21**, 197 (1957); **22**, 91, 98, 238 (1958).

33) L.E. Rhuland, *J. Bact.*, **73**, 778 (1957).

34) P. Meadow et al., *Biochem. J.*, **66**, 270 (1957).

Betaine is known as an intermediate metabolite of choline, which degrades through the path of betaine, dimethyl-glycine, sarcosine, reaching to glycine. The liberated methyl group are reported to make transmethylation reaction with various substances like homocysteine³⁵. However, the substances relating the path were all ineffective to *Ped. soyae* except betaine (Table III).

On the nutritional effects of amines, Herbst and Snell^{36,37} first reported that spermine, spermidine, putrescine or agmatine were essential growth factors for *Haemophilus parainfluenzae*. Momiyama and Miyaki³⁸ reported that putrescine, agmatine or arcaine were required by *Escherichia*, *Aerobacter* and *Salmonella*. That ethanolamine was effective for stimulating the growth of *Proteus morganii* was also reported by the same authors³⁹. Concerning the lactic acid bacteria, it was observed by Kihara and Snell⁴⁰ that the growth of *Lactobacillus casei* was markedly accelerated by the co-operation of streptogenin and spermine.

As for the nutrition of *Ped. soyae*, choline is quite ineffective because it is included as a constituent in the basal synthetic medium. Other 22 amines listed in Table IV were also not effective in inducing the growth of *Ped. soyae* when tested at the concentration of 10 mg/l.

V S-factors effects represented by betaine.

The fact that the supplementation of basal medium with betaine only resulted in the half-maximal growth offers an another problem whether the growth stimulating effect of P- or S-factor could be wholly attributed to that of betaine. In order to examine this, experiments were conducted as follows. *Ped. soyae* was grown in both 18% salted and unsalted media supplemented with P-factor (Polypepton) only, with

P-factor and betaine, with P-factor and S-factor (yeast extract), and with betaine only. Results given in Fig. 3 shows that maximal growth

TABLE III. EFFECT OF BETAINES AND ITS RELATED SUBSTANCES

	O.D.	N/20 NaOH ml
None	0.13	2.14
Betaine HCl 20mg/l	0.31	4.20
Lecithine 100mg/l	0.17	2.15
Acetyl choline chloride 20mg/l	0.14	2.30
Sarcosine 20mg/l	0.13	2.05
Procaine HCl 20mg/l	0.13	2.00
Putrescine 20mg/l	0.12	2.26
Dimethyl-glycine HCl 100mg/l	0.12	2.23
" 10mg/l	0.13	2.15
" 1mg/l	0.13	2.00
Dimethyl-glycine-methyl ester 100mg/l	0.13	2.18
" 10mg/l	0.12	2.05
" 1mg/l	0.13	2.22

After 17 days of incubation, the cell growth was assayed turbidimetrically and acidimetrically.

TABLE IV. EFFECT OF VARIOUS AMINES

	4 days	9 days
None	0.01	0.14
Betaine	0.45	0.47
Trimethylamine	0.01	0.15
Monoethylamine	0.01	0.14
Diethyl amine	0.01	0.15
Triethyl amine	0.01	0.15
Monoethanol amine	0.01	0.16
Diethylamino ethanol	0.02	0.14
Tetramethylammonium hydroxyde	0.02	0.06
β -phenyl-ethylamine	0.01	0.14
Dicyclohexyl amine	0.01	0.14
Tyramine HCl	0.01	0.15
Putrescine 2HCl	0.01	0.14
Cadaverine 2HCl	0.01	0.15
Histamine 2HCl	0.00	0.15
DL-Ornithine HCl	0.00	0.14
Agmatine H ₂ SO ₄	0.01	0.15
Tryptamine	0.01	0.15
Isoamylamine	0.00	0.15
Spermine	0.01	0.14
Spermidine	0.00	0.14
Ergothioneine	0.00	0.13
Creatine ^{41,42}	0.00	0.14
Creatinine	0.00	0.14
Mixture of all	0.00	0.00

The table shows the cell growth by optical density.

35) D.M. Greenberg, Chemical Pathways of Metabolism, vol. 2, 117 (1954).

36) E.J. Herbst and E.E. Snell, *J. Biol. Chem.*, **176**, 989 (1948); **181**, 47 (1949).

37) E.J. Herbst, E.B. Glinos and L.H. Amundsen, *Federation Proc.*, **11**, 229 (1952); *J. Biol. Chem.*, **141**, 47 (1955).

38) H. Momiyama, *Nippon Eiseigaku Zasshi*, **10**, 42 (1956); **11**, 291 (1957).

39) H. Momiyama, *ibid.*, **11**, 296 (1957).

40) H. Kihara and E.E. Snell, *Proc. Natl. Acad. Sci.* **43**, 867 (1957).

FIG. 3. S-factor Effect Represented by Betaine.

- x— Synthetic medium
 —Δ— +Polypepton 1.0%
 —○— +Polypepton 1.0% & yeast extract 0.3%
 ---Δ--- +betaine 30 mg/l
 ---○--- +betaine 30 mg/l Polypepton 1.0%

response was obtained by the addition of both P-factor and betaine as well as in the case of P-factor plus S-factor under the both environments of salted and unsalted media. Thus the effect of betaine is identical with that of S-factor. It was also noted that the single supplement with either betaine or P-factor to 18% salted media showed almost null growth even after thirty days of incubation.

VI. Existence of betaine in yeast extract and beef extract.

As S-factor rich substances, yeast extract, beef extract, heart infusion broth (Difco)³¹, unpasteurized soy sauce, and water extract of *Aspergillus sojae* mycelium¹¹ have been described in former reports. Sumi⁴³ reported the existence of a considerable amount (0.9 g/100 g) of betaine in spores of *Aspergillus*. Udo⁴⁴ in his laborious work on the constituents of soy sauce also found betaine with its sweet taste. Although betaine is widely distributed in plants⁴⁵, no information

has been reported on its presence in yeast extract and beef extract. It is also questionable that the growth promoting substance in yeast extract or beef extract is only betaine.

The experiments were conducted chromatographically to identify the biologically active spot and the spot of betaine on paper. Yeast extract and beef extract (Difco) were extracted with ethanol, the soluble fraction and the residue were each spotted on the lower tips of paper strips (Tōyō Rōshi No. 53) and developed for about thirty hours at 20°C using the solvent of *n*-butanol, acetic acid and water mixed in the ratio of 4:1:2. To detect the microbiologically active spot, the developed and dried paper strip was cut in each 1 cm, put into cotton plugged test tubes, poured by the 5 ml of basal synthetic medium, and their growth promoting activities were assayed as usual. The colour spot of tertiary amines and tetrarary ammonium salts including choline and betaine was detected by spraying Dragendorff's reagent. The samples added with betaine were also chromatographed to calibrate the R_F of betaine in crude mixtures.

41) R.H. Nimmo-Smith and G. Appleyard, *J. Gen. Microbiol.*, **14**, 336 (1956).

42) G. Appleyard and D.D. Woods, *ibid.*, **14**, 351 (1957).

43) M. Sumi, *J. Agr. Chem. Soc. Japan*, **2**, 866 (1925).

44) S. Udo, *ibid.*, **7**, 852 (1931); **8**, 675 (1932).

45) M. Guggenheim, *Die Biogenenaminen*, p. 193 (1940).

FIG. 4. Bioautograms of Yeast Extract, Beef Extract and Betaine-HCl, and their Paper Chromatograms Sprayed by Dragendorff's Reagent.

Fig. 4 shows that the growth stimulants are rather ethanol soluble, Dragendorff positive spots and the spot of mixed betaine are included in the area of biologically active spots. The active spots which do not coincide with the spots of tertiary amines may be partly due to the action of contaminating P-factor. Such phenomena is rather distinct in the case of yeast extract. In general, at least a part of the S-factor substances in yeast extract or beef extract are attributable to betaine contained in them.

DISCUSSION

Pediococcus soyae is a very specific lactic acid bacterium in the respect of its high osmotolerant character growing even in 24~26% salted media⁴¹. This organism grows only very poorly in the ordinary synthetic medium employed for bioassay⁵¹, and requires two different specific growth factors for maximal growth. One of them, provisionally named as S-factor in former reports, is a constituent of Difco's yeast extract, beef extract, heart infusion broth, unpasteurized Japanese soy sauce, etc.. In this report it is elucidated that the action of S-factor was perfectly replaced by the addition of betaine hydrochloride 30 mg/l. It was therefore observed that at least one of the active constituents in the yeast extract or beef extract was betaine. However, the problem that some other growth stimulating substances might exist in them is still remained to be clarified. The optimum concentration of betaine hydrochloride as high as 30 mg/l also sustains this question because it is too high as compared with the required amounts of ordinary vitamins and it almost nears to the level of amino acids or other substrates.

Betaine is a usual component in plants⁴⁵, and

it is expected that the substance may play some roles in their metabolism and physiology. It has been known that betaine is an intermetabolite of choline, and it reacts as a substrate of trans-methylation. It has an effect as a lipotropic factor for the prevention of animal fat liver, and also as a growth factor substitutable with choline. However, betaine is useless, in contrast to choline, for the prevention of chick's perosis and for its growth stimulation^{46,47}. As for the effects of betaine against *Ped. soyae*, the growth promoting effect and the osmotolerancy bestowing effect, are the unique faculty of betaine because no other metabolically related substances such as choline, dimethyl glycine, sarcosine or methionine are effective. It is supposed from these facts that betaine is effective for *Ped. soyae* not through the metabolic path ever known, but may be directly effective for maintaining the semi-permeability of the cell membrane in co-operation with P-factor.

The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara of the Institute of Applied Microbiology, University of Tokyo for their kind guidances. He is greatly indebted to Prof. K. Miyaki and Assist. Prof. M. Hayashi of Chiba University for their kind advices and gift of dimethylglycine. Generous supply of many precious vitamins and growth factors were made by Assist. Prof. G. Tamura and Mr. T. Fukami of University of Tokyo. The author's thanks are also due to the kind advices given by Dr. Mogi and members of this institute. He is especially grateful to Mr. T. Kuramochi for his earnest and able assistance.

⁴⁶ A.D. Welch, *J. Nutr.*, **40**, 113 (1950).

⁴⁷ T.H. Jukes and A.D. Welch, *J. Biol. Chem.*, **146**, 19 (1942).