MICROBIAL PRODUCTION OF AMINO ACIDS III. NUTRITIONAL STUDIES AND THE EFFECTS OF ORGANIC GROWTH PROMOTERS AND THIAMINE ON DL-ALANINE FERMENTATION BY *Arthrobacter* STRAIN $C_{19}d$

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ABSTRACT

The study of the nutritional requirements of Arthrobacter *strain $C_{19}d$ which accumulates alanine in large amounts in the culture medium, revealed that the organism needs thiamine for its growth. Also the alanine accumulation by this strain was found to be related to thiamine concentration in the medium. The optimum concentration of thiamine for alanine accumulation (20 μg/ml) was also optimum for the growth of the organism indicating thereby that alanine accumulation by this strain is a growth associated process rather than far removed from it. Among the various growth promoters tried yeast extract was found to be superior from the point of view of alanine yield and it was also superior to giving thiamine alone in the medium. A concentration of 0.02% yeast extract was found to be optimum for alanine occumulation.*

INTRODUCTION

By and large, amino acid fermentations are controlled by singled out or mixture of growth factors which the microorganisms demand for their growth. Generally only suboptimal levels of these growth factors are needed for accumulation of amino acids in large amounts in the culture broths. As such use of auxotrophic microorganisms has turned out to be the key to success in amino acid fermentations.

Though alanine is one of the simplest, nonessential amino acids often found in appreciable amounts in the culture broths of various types of microorganisms, its accumulation appears to be controlled by the nutrients, particularly thiamine, the organism demands for its growth. Earlier, Tanaka and Kinoshita (1960) in their studies with *B. lentus* pointed out that thiamine may play an important role in alanine accumulation. Later Ogata *et al.* (1969) made similar observations in their studies with a psychrophilic

strain of *Brevibacterium*, accumulating alanine in the medium. In both the above cases, alanine accumulation was related to the thiamine requirement of the strain and suboptimal levels of thiamine in the medium, was found to increase the alanine accumulation. Our results also indicate the positive role of thiamine in the accumulation of alanine by this *Arthrobacter* strain $C_{19}d$.

In an earlier communication we have detailed the conditions required by this strain $C_{19}d$, for alanine production in a synthetic medium containing glucose and urea[5]. This communication presents the nutritional requirements of this organism and the effects of some of the organic growth promoters and particularly the role of thiamine, on alanine production by this strain.

Materials and Methods

The *Arthrobacter* strain $C_{19}d$ used in the present investigation was isolated from soil, using glycine enrichment[3]. The basal medium which was supplemented with organic growth promoters as each experiment demanded, contained, glucose 75.0 *g*; urea 5.0 *g*; K_2HPO_4, 0.5 *g*; KH_2PO_4, 0.5 *g*; $MgSO_4.7H_2O$, 0.25 *g*; $MnSO_4\ H_2O$ and $FeSO_4\ 7H_2O$, 0.01 *g* and distilled water 1000 *ml*, adjusted to *pH* 7.3.

Erlenmeyer flasks (500 *ml*) containing 100 *ml* of the above medium were inoculated after sterilization at 121°*C* for 20 min. Glucose was always sterilized separately at 116°*C* for 15 min and urea was filter sterilized and added to the medium at the time of inoculation. The flasks were incubated on a rotary shaker (250 r.p.m.) at room temp (24 – 28°C). During cultivation the *pH* of the medium was kept between 7 ~ 8.

Alanine was estimated by paper chromatography according to the method described by Giri *et al.*[2]. Bacterial growth was measured turbidimetrically with a Bausch & Lomb colorimeter. The culture broth was diluted twenty-fold and its optical density was measured at 540 $m\mu$.

The vitamins used in the study were obtained from B.D.H. Laboratories, Chemicals Division, Bombay. The organic growth promoters like peptone, beef extract, yeast extract, nucleic acids (yeast), vitamin-free casamino acids were of either Difco or Oxoid preparations.

Result and Discussion

Growth Requirements of the Organism :

A preliminary identification of the growth factor requirements of the organism was carried out using organic growth promoters like vitamin-free casamino acids, synthetic vitamin mixture, nucleic acids and yeast extract as sources of amino acids, vitamins and purines and pyrimidines, respectively.

The results of the experiments shown in Table I-A, revealed that this organism could grow well wherever vitamin mixture either singly or in combinations of other growth promoters was supplied. It could grow equally well on yeast extract also. However, casamino acids and nucleic acids could not promote the growth either singly or in combination, since the increase in growth was not significant as compared to the control. The fact that vitamin mixture alone could give as much growth as the one in which all the organic growth promoters were combined, was suggestive of the essentiality of a particular vitamin or vitamins, for the growth of this organism. So the vitamin requirement of this organism was studied by the vitamin exclusion technique on the lines followed by Dias and Bhat (1963), the results of which are shown in Table I-B, It is clear from the results of this experiment that the organism needs only thiamine for its growth.

TABLE I A & B

Growth Requirements of C_{19} *d*

I-A

Growth factors added	Growth as O.D.
Vitamin free casamino acids (VFCAA).	0.05
Vitamin mixture.	0.17
Nucleic acids	0.06
VFCAA + Vit. mixture	0.17
VFCAA + Nucleic acids	0.05
Vitamin mixture + nuclic acid.	0.17
VFCCA + Vit. mixture + nucleic acid	0.17
Yeast extract	0.17
None added	0.04

I-B

Vitamins excluded	Growth as O.D.
None	0.07
Pyridoxine	0.07
Thiamine	0.00
PABA	0.07
Nicotinic acid	0.08
Riboflavin	0.08
Ca.panthothenate	0.08
Biotin	0.08
Folic acid	0.07
Vitamin B_{12}	0.08
All	0.00

Growth—5 days

Vitamin mixture: Pyridoxine thiamine, PABA, Nicotinic acid Riboflavin, Calcium Panthothenate Biotin, Folic acid, and Vit.B12 growth—96 hr.

Effect of organic growth promoters on alanine production:

The effect of various organic growth promoters like peptone, beef extract and yeast extract on alanine production was then tested, with a view to increase the yield of alanine. The results are presented in Table II.

TABLE II

Effect of Organic Growth Factors on Alanine Production by C_{19} *d*

Nutrient added	Levels of nutrient added%	Growth in (O.D.) Alanine *mg/ml*	Incubation in hours			
			25	36	48	60
Peptone	0 05	*pH*	8.2	8.2	8.2	8.0
		Growth	0.12	0.19	0.21	0.31
		Alanine	....	0.80	1.20	1.50
	0.10	*pH*	8 2	8.2	8.2	7.0
		Growth	0.17	0.25	0.33	6.46
		Alanine	.....	1.2	2.0	2.7
Beef extract	0.05	*pH*	8.0	8.2	8.2	8.5
		Growth	0.11	0.16	0.17	0.25
		Alanine	neg	0.6	1.1	1.0
	0.10	*pH*	8.0	8.2	8 2	7.3
		Growth	0.17	0.24	0.26	0.42
		Alanine	neg	1.1	1 6	1.7
Peptone + Beef Extract	0 05	*pH*	8.2	8.2	8.2	7.0
		Growth	0.17	0.25	0.33	0.46
		Alanine	neg	1.2	2.0	2.7
	0.10	*pH*	8.2	8.0	8.0	6.7
		Growth	0.23	0.3	0.62	0.75
		Alanine	neg	1.7	2.5	2.7
Yeast extract	0.05	*pH*	8.2	8.2	8.2	8.0
		Growth	0.23	0.35	0.42	0.50
		Alanine	.....	1.7	2.8	3.5
	0.10	*pH*	8.2	8.2	7.0	6.4
		Growth	0.30	0 52	0.78	0.82
		Alanine	1.1	3.2	2.8	2.1

– = negligible

Product formation was good with all the three growth promoters provided in the medium either singly or in combination. Without any growth factors, the organism accumulates only negligible amounts of alanine and the growth also will be scanty. Among the three growth promoters examined, yeast extract was superior to other two from the point of view of growth as well as alanine formation. Combination of peptone and beef extract did not prove better than either peptone alone at 0.10% level or yeast extract at 0.05% level. However, growth was better with this combination than with peptone and beef extract individually and was in a way comparable with that of yeast extract at both levels. Since yeast extract was superior to other two growth promoters examined, various levels thereof were put to the test in order to arrive at the optimal concentration required for maximal product formation. The results of the experiment are shown in table III.

Lower levels of yeast extract were found to be highly stimulatory and adequate for product formation; higher levels were conducive only to the growth of the organism rather than product formation. As can be seen from the table, yeast extract at 0.02% level was far superior and raised the yield of alanine to 5.6 *mg/ml* which is nearly 50 per cent more than the previous best yield (3.5 *mg/ml*).

Effect of thiamine on alanine accumulation by C_{19} d :

Since the organism needs thiamine for its growth, the effect of various levels of thiamine on alanine accumulation was examined. Table IV shows the results of the experiment.

Growth of the organism as well as the alanine accumulation were found to be closely related to the concentrations of thiamine provided in the medium. Apart from this glutamic acid accumulation which used to be in negligible amounts, also was stimulated considerably by thiamine. It can be seen from Figure 1 that the thiamine concentrations upto 10 μ *g/ml* were stimulative for glutamic acid accumulation, whereas for alanine upto 20 μ *g/ml* concentration was, in fact, required. This experiment was indicative of the optimum levels of thiamine needed for glutamic acid and alanine accumulation respectively. The yields of glutamic acid and alanine at their respective optimum levels of thiamine were raised to 3.5 *mg/ml* and 7.6 *mg/ml* respectively. Also the optimum level of thiamine needed for maximal alanine production was optimum for growth of the organism indicating that the product formation is essentially growth associated rather than far removed from it. The low values obtained for growth and amino acids (Table IV) in the treatment receiving 5 μ *g/ml* thiamine, up to 48 hr. growth, are due to frequent *pH* corrections made during that period as the *pH* tended to shift towards acid side.

TABLE III

Effect of Yeast Extract on Alanine Production by $C_{19}d$

Nutrient added	Amount added %	Growth (O.D.) Alanine (mg/ml)	Incubation in hours 48	72	96
Yeast extract		pH	8.2	8 2	8.2
	0.005	Growth	0.41	0.41	0.43
		Alanine	3.6	3.8	2.5
		pH	8.2	8.0	6.0
	0.01	Growth	0.44	0.54	0.55
		Alanine	4.6	4.8	3.3
		pH	8.2	7.3	7.0
	0.02	Growth	0.41	0.54	0.61
		Alanine	5.6	5.6	3.6
		pH	8.2	8.2	8.0
	0.05	Growth	0.42	0.75	0.75
		Alanine	2.8	3.5	4.0

Basal medium with glucose 7.5%, urea 0.5%.
Boitin 2.5 $\mu g/Ll$ inoculum 1%.

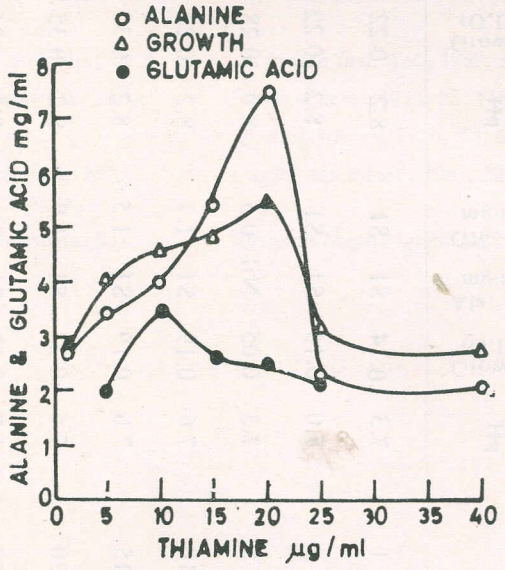

FIG. 1

Effect of Thiamine on Alanine formation by C_{19} *d*

TABLE IV

Effect of Thaimine on Alanine Production by $C_{19}d$

Thiamine added µg/ml	Incubation Time: 24 hrs pH	24 hrs Growth (O.D)	24 hrs Ala. mg/ml	24 hrs Glu. mg/ml	48 hrs pH	48 hrs Growth (O.D)	48 hrs Ala. mg/ml	48 hrs Glu. mg/ml	72 hrs pH	72 hrs Growth (O.D)	72 hrs Ala. mg/ml	72 hrs Glu. mg/ml	96 hrs pH	96 hrs Growth (O.D.)	96 hrs Ala, mg/mi	96 hrs Glu. mg/ml
1	7.3	0.14	Sl	Sl	8.2	0.22	1.1	2.0	7.5	0.24	1.3	2.6	6.4	0.25	1.5	3.1
3	7.0	0.15	Sl	Sl	8.2	0.22	1.4	1.5	8.2	0.27	2.1	2.5	8.2	0.27	2.7	2.5
5	7.3	0.05	Nil	0.5	8.0	0 28	Nil	0.7	8.2	0.35	2.5	2.0	7.9	0.42	3.4	1.3
10	7.6	0.19	Sl	1.7	8.2	0.28	2.6	3.5	8.0	0.37	4.6	3.5	8.0	0.46	4.0	3.0
15	7 6	0.19	Sl	1.5	8.2	0.30	3.0	2.6	7.9	0.38	5.6	2.3	7.9	0.48	5.4	2.3
20	7.6	0 22	Sl	1.9	8.2	0.35	3.2	2.5	7.9	0.47	7.6	2.1	7.9	0.55	6.4	2.1
25	6.7	0.15	Sl	Sl	8.2	0.26	1.1	1.7	8.2	0.30	2 0	0.8	8.2	0.32	2.3	0.7
30	7.0	0.17	Sl	Sl	8.2	0.27	1.1	1.6	8.2	0.31	1.8	0.8	8.2	0.32	2.1	0.7
40	6.7	0.17	Sl	Sl	8,2	0 25	1.1	1.6	8.2	0.27	2.5	0.7	8.5	0.28	2.5	0.7

Basal medium with glucose 10.0%, urea 0.75%, inoculum 10%
Sl = slight

Stimulatory effects of thiamine on glutamic acid accumulation has been reported by Takahashi *et al.* (1965). Their organism, a *Corynebacterium* spp. responded to thiamine upto 10 $\mu g/1$. However, the difference in amounts of thiamine needed for maximum accumulation of glutamic acid by their strain and C_{19} *d* is quite wide. Besides the optimum amounts of thiamine needed for alanine accumulation by the latter strain (20 $\mu g/ml$) is rather high as compared to that (1 $\mu g/ml$) reported by Ogata *et al.* (1969). Higher levels of thaimine above 20 $\mu g/ml$, however reduced the yields of alanine markedly (Table IV) though growth was only slightly affected and the *pH* remained at optimum indicating the probable role of thiamine in alanine accumulation.

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