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Nutritional Reguirements for Growth of *Cellulomonas flavigena* on cellulosic substrates

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Cellulose 基質에서 *Cellulomonas flavigena*의 生長에 대한 營養要求性

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ABSTRACT

Nutritional requirements for the growth of *Cellulomonas flavigena* were studied. *C. flavigena* grew well on cellulose when 0.005% or more of yeast extract was present in the growth medium. The growth factor in yeast extract was, in part, thiamine and biotin. Amino acids had little effect on the growth on the organism. The extent of growth on yeast extract was much higher than that obtained on those vitamins, which indicates the presence of growth factors in yeast extract besides the vitamins. among the carbohydrates tested, the organism grew best on glucose and galactose, and the optimum N/P ratio was within the range of 0.75~3.17

INTRODUCTION

Cellulosic crop residue is the major agricultural waste and is not effectively utilized. Because of the need for waste utilization and pollution abatement, considerable efforts have been made to utilize these materials. Production of single cell protein from cellulosic materials is an example (Han, *et al* 1971; Bellamy, 1975; Thayer, *et al* 1975). The development of a good medium is crucial to the yield of cell mass which is the single most important economic factor in SCP production. The medium supplies nutrients for growth, energy, building materials of cell constituents, and biosynthesis of fermentation products. A poor choice of medium components causes limited cellular growth and affect the type and yield of fermentation products. Cellulosic substrates, such as crop residues, contain limited nutrient for microbial growth except for the carbon and energy source which is provided by the cellulose. All other nutritive elements must be supplied exogenously. Limitation of any one of the essential nutrient will retard the cell growth while excess of other nutrients will not only add cost but also detrimental to microbial growth.

Therefore, the optimal level of each nutrients for the maximum cell growth should be carefully determined.

This paper reports the results of nutritional studies for the growth of *Cellulomonas flavigena* on cellulosic substrates.

Materials and Methods

1. Microorganisms, media, and growth

The cellulose digesting microogranisms used in this study were *Cellulomonas flavigena* (ATCC 21399) and *Alcaligenes faecalis* (ATCC 21400), and their characteristics have been reported elsewhere (Han and Srinivasan, 1968; Han and Srinivasan, 1969). The composition of basal medium was $(NH_4)_2SO_4$, 6.0g; KH_2PO_4, 1.0g; K_2HPO_4, 1.0g; $MgSO_4 \cdot 7H_2O$, 0.1g; $CaCl_2$, 0.1g; yeast extract, 0.5g; and 10g of sodium carboxymethyl cellululose per liter of water. Different kinds and levels of carbon, nitrogen, phosphate and minerals were substituted in the basal medium to study their effect on growth of the organisms. The cells were grown in Erlenmyer flasks or T-formed shake tubes (Belco Co) on a reciprocal shaker at 35°C. The cell concentration was determined with a Spectronic 20 colorimeter(660nm) or a Klett colorimeter(green filter). Dry weight of cell mass was determined by filtering the cultue on millipore filter(0.45μ) and drying at 95°C to constant weight.

2. Vitamins and amino acid requirement

Twenty different media were prepared in duplicate. One consisted of the basal medium plus 0.05% yeast extract, another consisted of the basal medium plus 5 ppm each of 17 amino acids to be tested. and a third was the basal medium from which yeast extract was deleted. The remaining 17 broths consisted of 5ppm each of 16 of the 17 amino acids, deleting 1 amino acid at a time. In a different set of experiment, a vitamin mixture containing 5 ppm each of 11 vitamins was added to the broth that contained a particular amino acid to be tested. The 17 amino acids and 11 vitamins tested were listed in Table 3 and 4.

The broths were prepared in T tubes and a loopful of cell suspension was inoculated. Actively growing culture

Table 3. Amino acid and vitamin requirement for growth of *C. flavigena*

Amion acid and vitamin	Growth (OD 660 nm)
Yeast extract	0.41
17 amino acids	0.01
No amino acid	0.02
17 amino acino acids and 11 vitamins	0.27
No amino acids & 11 vitamins	0.28
Methionine [a] & 11 vitamins	0.28
Glycine – & 11 vitamins	0.27
Leucine – & 11 vitamins	0.26
Proline – & 11 vitamins	0.18
Cystine– & 11 vitamins	0.31
Asparagine – & 11 vitamins	0.27
Glutamic acid – & 11 vitamins	0.24
Histidine – & 11 vitamins	0.27
Tryptophan – & 11 vitamins	0.32
Phenylalanine – & 11 vitamins	0.29
Arginine – & 11 vitamins	0.24
Serine – & 11 vitamins	0.27
Tyrosine – & 11 vitamins	0.26
Lysine – & 11 vitamins	0.27
Valine – & 11 vitamins	0.33
Threonine – & 11 vitamins	0.28
Isoleucine – & 11 vitamins	0.25

[a] The particular amino acid was deleted from the 17 amino acino acid mixture.

Table 4. Vitamin requirement for growth of *C. flavigena*

Vitamin	Growth [a]			
	1 day	2 days	3 days	4 days
Control (basal medium)	− −	− −	− −	− −
Yeast extract	− −	+ +	+ +	+ +
Biotin	− −	− −	+ +	+ +
I-inositol	− −	− ±	− ±	− ±
Riboflavin	− −	− −	− −	− −
Niacinamide	− −	− ±	− ±	− ±
Thiamine. HCl	− −	− ±	+ +	+ +
Ascorbic acid	− −	− −	− −	− −
Pantothenic acid	− −	− −	− −	− −
Folic acid	− −	− ±	− ±	− +
Vitamin B_{12}	− −	− −	− −	− −
Cholin chloride	− −	− −	− −	− −
VitaminB_6 HCl	− −	− −	− −	− −
Biotin + B_6 HCl	− −	− −	+ +	+ +
Riboflavin+B_6 HCl	− −	− −	− −	− −
Thiamine + B_6 HCl	− −	+ +	+ +	+ +
Pantothenic acid + B_6 HCl	− −	− −	± +	± +
Biotin + Riboflavin + B_6 HCl	− −	+ +	+ +	+ +
Thiamine HCl+pantothenic acid+B_6 HCl	− −	− +	+ +	+ +
Biotin + Riboflavin + Thiamine + Pantothenic acid B_6 HCl	− −	+ +	+ +	+ +

[a] Growth was observed by turbidity in duplicated tubes;
−, No growth (Kletts unit less than 5)
±, Klett unit 5—50
+, Growth (Kletts units more than 50)

(48hr)of the test organisms were washed twice by centrifugation and resuspending in distilled water before used as inoculum.

Results and Discussion

Table 1 shows the effect of different carbon source on the growth of *C. flavigena*. The highest cell yield was obtained on glucose and galactose that produced about 0.3g dry cell per liter during the 32 hr incubation period. There was little growth on methyl- and hydroxyethyl-cellulose. Monosaccharides (glucose and galactose) were better utilized than the disaccharides (cellobiose, maltose and lactose).

In the case of single-cell-protein production from cellulosic substrates, carbon source other than cellulose is of little concern. When *C. flavigena* was grown on a mixture of soluble and insoluble carbohydrates the organism preferentially utilized the former and then attacked the latter (Callihan and Dunlap, 1973). When *C. flavigena* was grown on insoluble cellulosic substrate certain level of

Table 1. Effect of different carbon source on the growth of *C. flavigena*

Carbon source	Cell growth (Klett Unit)	Cell mass (g/l)
Glycerol	75	0.098
Glucose	210	0.285
Galactose	230	0.300
Cellobiose	180	0.235
Maltose	120	0.155
Lactose	155	0.202
Carboxymethylcellulose	193	0.250
Methylcellulose	40	0.052
Hydroxyethylcellulose	61	0.079

a The organism was grown for 32 hr in a basal medium containing 1% of the different carbon source.

initial soluble carbohydrate was necessary to promote the cell population and to reduce the lag period. Excess souble carbohydrate, especially cellobiose was reported to be inhibitory for cellulase synthesis (Reese, 1956). *C. flavigena* grew well on cellulose especially in the presence of *A. faecalis*. This is believed to be a symbiotic effect of the both organisms: *C. flavigena* and *A. faecalis*.

The former produces cellobiose from cellulose and the latter eliminates, the inhibitory cellobiose from the culture medium. The overall effect would be facilitation of cellulose hydrolysis and promotion of cell growth.

A. faecalis produced β-glucosidase when grown on lactose(Han and Srinivasan, 1969). Therefore, lactose is the preferred carbon source for symbiotic cultivation of *C. flavigena. and A. faecalis.*

Table 2 shows the effect of nitrogen and phosphate levels on the growth of *C. flavigena.* Maximum cell growth was observed in the media that contained

Table 2. Effect of N and P levels and N/P ratio on the growth of *C. flavigena*

$(NH_4)_2SO_4$ (%)	Potassium phosphate [a] (%)	N/P ratio	Cell growth (Klett nuit)
0	0.4	0	0
0.1	0.4	0.25	260
0.3	0.4	0.75	290
0.6	0.4	1.5	290
1.0	0.4	2.5	272
0.6	0	∞	43
0.6	0.02	31.75	109
0.6	0.05	12.70	218
0.6	0.10	6.35	239
0.6	0.20	3.17	250
0.6	0.40	1.58	290
0.6	1.00	0.63	218

[a] Equal part of K_2HPO_4 and KH_2PO_4 were mixed to make up the concentration.

0.3—0.6% $(NH_4)_2SO_4$ and 0.2—0.4% potassium phosphate. Low level of N and P resulted in a limited cell growth while high level of these elements did not retard the cell growth, except that 1% phosphate slightly reduced the cell growth. Excessive phosphate might have tied up Fe^{++}, Mg^{++}, Ca^{++} and other essential metals. The optimum N/P ratio in the growth medium was within the range of 0.75—3.17.

It is conceivable, however, that the initial N/P ratio in the batch medium will soon become changed as the organisms grow and utilize a certain fixed N/P ratio. Thus, determination of an optimum N/P ratio in a batch culture medium would be more critical than that in a continuous culture, because the ratio would be changed in the former while the ratio stays the same in the latter. If a proper N/P ratio is not maintained in a batch culture, one nutrient might

soon become exhausted while other elements become excessive and inhibitory to the microbial cell growth.

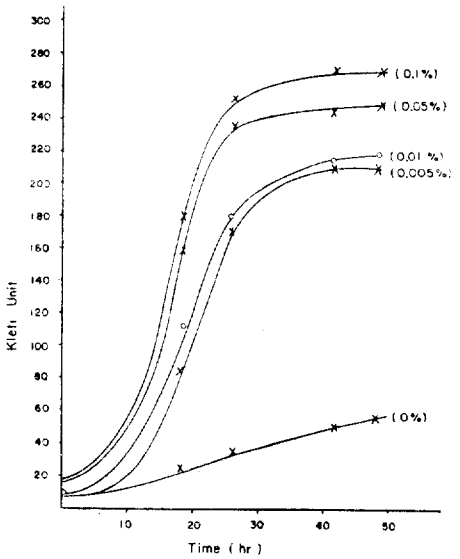

Fig. 1. Effect of yeast extract on the growth of *C. flavigena*

C. flavigena required yeast for its growth as shown in Fig 1. The required amount of yeast extract varied depending upon the size of the inoculum; larger inoculum required lesser amount of yeast extract. This is probably due to the autolyzed cells which provides the growth factors. Thus, minimal requirement of yeast extract for growth is difficult to assertain without specifying the inoculum size.

In order to identify the essential components in yeast extract 17 amino acids and 11 vitamins were selected and tested for their ability to support the growth of the organism. Table 3 shows the growth responce of *C. flavigena* to different amino acids and vitamins. The organism grew well on media that contained yeast extract but it did not grow when the yeast extract was replaced by 5 ppm each of 17 different amino acids. However, the organism grew well when water-soluble vitamins were added to the amino acid containing media whether it contained all or 16 amino acids from which one particular amino acid was deleted. Thus, it is apparent that the growth promoting factor in yeast extract is among certain vitamins but not among the amino acids.

As Table 4 shows the medium containing thiamine or biotin supported the growth of the organism. Any combination of vitamins that included thiamine or biotin also supported the growth of the organism. Effect of niacin, i-inositol, and folic aicd were not conclusive because growth response was not consistent on these vitamins. Even though thiamine and biotin were essential for the growth, deletion of these vitamins from the 11 vitamin mixture did not affect the growth of the organism. Apparently presence of other vitamins complemented these two vitamins. The growth on a medium in which yeast extract was replaced by any one

Table 5. Effect of trace minerals on the growth of *C. flavigena*

Trace mineral solution [a] (ml/l of basal medium)	Cell growth [b] (Klett unit)
0	216
0.1	219
0.5	228
1.0	230
5.0	210
10.0	26

[a] Mineral solution contains $CaCl_2$, 0.5g; $FeCl_3 \cdot 6H_2O$, 16.7g; $ZnSO_4 \cdot 7H_2O$, 0.18g; $CuSO_4 \cdot 5H_2O$, 0.16g; $CoCl_2 \cdot 6H_2O$, 0.18g; EDTA, 20.1g; H_2O, 1 liter.

[b] *C. flavigena* was grown on basal medium for 26 hr on a reciprocal shaker.

of the amino acid, vitamin, or a combination of these did not reach that obtained by addition of yeast extract. Thus, it appears that yeast extract contains growth factor(s) for *C. flavigena* other than vitamins tested.

Effect of trace minerals on the growth of *C. flavigena* is shown in Table 5. There was no difference in cell yield among the media that contained 0 to 5 ml of mineral soluton. However, apparent inhibition of cell growth occurred on a medium containing 10ml of mineral solution. Most of the microorganisms require minerals for their growth. But, these minerals are required in such a small amount that these trace minerals are easily supplied by other medium components. Thus, it is difficult to determine exact amount of mineral requirement. The inhibition of cell growth by addition of a large amount (10ml) of mineral soultion may have been due to excess amount of EDTA which might chelate the metals in various enzymes.

摘 要

本實驗은 *Cellulomonas flavigena*의 生長에 필요한 營養 要求性에 대하여 연구하였다.

*C. flavigena*는 cellulose 培地에 酵母 extract를 0.005% 或은 그 以上 添加하였을 때 增殖을 잘 하였다. 酵母 extract 內의 生長要因은 部分的으로 thiamine과 biotin이였고 amino acids는 거의 영향을 주지 않았다. 酵母 extract에서의 增殖程度는 vitamin을 주었을 때 보다 훨씬 높았는데 이것은 vitamins 外에 酵母 extract內에 他生長要因이 存在하고 있음을 시사해 준다.

試驗한 炭水化物中에서는 포도당과 유당에서 가장 좋았고 最適 N/P比는 0.75~3.17 범위 내에 있었다.

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