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Enzymatic Reactions in Citric Acid Fermentation of Mandarin Orange Peel by *Aspergillus niger*

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만다린 오렌지 과피를 기질로 한 *Aspergillus niger*의 구연산 발효에 관련된 효소적 반응

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Abstract — When mandarin orange peel was used for a substrate of citric acid fermentation by *Aspergillus niger*, principal enzyme activities were investigated. Not only the activity of polygalacturonase and pectin esterase being capable of digesting pectin and crude fiber of mandarin orange peel, but also that of carboxymethyl cellulase, xylanase and amylase was high. In carbohydrate metabolism, the activity of enzymes related in HMP pathway was higher than that in EMP pathway at the orange peel medium designed hereby rather than synthetic medium. Productivity of citric acid was significantly increased when the activity of citrate synthetase was high and simultaneously those of aconitase and NADP-dependent dehydrogenase were low.

Citric acid has been produced industrially from carbohydrate such as glucose, molasses and starch by a fermentation processing using a strain of *Aspergillus niger*. The accumulation of extracellular citric acid by fungi was discovered by Wehmer in 1893 and most of the commercial citric acid today are produced by fermentation with *Aspergillus niger* (1). Up to now, the mechanism of citric acid accumulation from carbohydrates by a strain of *Aspergillus niger* has been discussed from the viewpoint of change in enzyme activities relating to the tricarboxylic acid cycle. It is generally believed that there must be an inhibition of tricarboxylic acid cycle, probably on the level of isocitric dehydrogenase or α-ketoglutarate dehydrogenase while there should be an undisturbed metabolic flow through glycolysis. But there is little known about citric acid fermentation with crude carbohydrate except glucose and sucrose (2). In this report, we describe the pathway of citric acid accumulation and glycolysis by examination of principal enzyme activity concerned with citric acid fermentation by isolated strain *Aspergillus niger* GFM-0014 using mandarin orange peel as a crude carbohydrate.

Key words: Orange peel, citric acid fermentation, *Aspergillus niger*
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Materials and Methods

Strain and media

Aspergillus niger GFM-0014 isolated from mandarin orange peel was used (3). Orange peel medium (orange peel powder 60 g in 1 L of distilled water) and synthetic medium (Sucrose 150 g, NH_4NO_3 20 g, KH_2PO_4 10 g, $MgSO_4 \cdot 7H_2O$ 2.5 g in 1 L of distilled water) were used for cultivation. Mandarin orange peel was dried at 70~90℃ (moisture content 12%) in hot air tunnel type dryer and finely ground. Enzymes and reagents used for the determination of enzyme activity were commercially purchased (Si-

gma Co. USA).

Cultivation

For cultivation, spore suspension (1×10^8 spores /m*l*) was prepared after incubation of *Aspergillus niger* GFM-0014 on potato dextrose agar slant at 30℃ for 10 days. Submerged culture was carried out in orange peel medium at 30℃, 120 rpm for 7 days in 500 m*l* shaking flask.

Preparation of cell free extracts

Activity of enzyme related to glycolysis and citric acid synthesis was determined. Cells cultured were separated and washed. One gram of sea sand was added to 1 g of dry cell, followed by disintergration at 4℃. 10 m*l* of 0.05 M phosphate buffer (pH 7.4) was added, mixed, and then centrifugated (10,000 rpm. 10 min). Supernatant was used as cell free extract. Enzyme activity was assayed at 25℃ with UV-visible spectrophotometer (Shimadzu UV-260, Japan).

Measurement of enzyme activity

Polygalacturonase was measured by method of Toshio (4). Pectin esterase was assayed by method of Ueda (5). CMCase and xylanse was determined according to method of Horikoshi (6). Hexokinase, phosphofrucokinase pyruvatekinase, glucose-6-phosphate dehydrogenase and transketolase were assayed by methods of Caltrider (7), Ling (8), Bucher (9), Kornberg (10) and Horecker (11), respectively. One unit of enzymes was defined as the amount of enzyme which hydrolyzed 1 μmole of substrate $ml^{-1}\cdot min^{-1}$.

Principal enzymes relating to synthesis of citric acid in TCA cycle

Citrate synthetase, aconitase and NAD/NADP dependent isocitrate dehydrogenase were measured by method of Srere (12), Anfinsen (13) and Kornberg (10), respectively.

Results and Discussion

To obtain the information of cirtic acid accumulation from the fermentation of mandarin orange peel by *Asp. niger*, activities of key enzymes in glycolysis and TCA cycle were investigated.

Table 2 shows the activities of pectin esterase, polygalacturonase, CMCase, xylanase and amylase produced by *Asp. niger* GFM-0014. Pectinesterase and polygalacturonase show high activities in both synthetic and orange peel media, in spite of different amount of pectin in medium. But the activties of these enzymes were increased as the amount of pectin in media increased, and it seemed that most of pectin in mandarin orange peel (25.3%) was digested by these enzymes and converted to another metabolic pathway. This suggested a close relationship between the activity of extracellular pectin digestable enzymes and accumulation of citric acid, although some extent can be varied according to medium composition used (15).

Fig. 1 and 2 show the effect of media and culture

Table 1. Chemical composition of mandarin orange peel

Components	Contents% (W/W)	Components	Contents% (W/W)
Moisture	12.4	Crude fat	1.3
Carbohydrate	78.5	Citric acid	1.14
Total sugar	34.4	Potassium	0.64
Reducing sugar	14.1	Calcium	0.20
Pectin	25.3	Magnesium	0.03
Hesperidin	5.2	Iron	0.012
Naringin	2.9	Copper	Trace
Crude fiber	4.3	Zinc	Trace
Crude protein	4.8	Manganese	Trace
Crude ash	1.5		

Table 2. Activities of enzymes produced by *Aspergillus niger* GFM-0014 in the various submerged cultures

Medium[a]	Mycellium Dry weight (mg/50 ml)	Protein content (mg/ml)		PG (units)		PE (units)		Amylase (units)		CMCase (units)		Xylanse (units)	
		CFE	CE	CFE	CE	CFE	CE	CFE	CE	CFE	CE	CFE	CE
A	1,554	0.36	0.42	0.94	4.13	0.18	0.41	0.42	0.44	0.04	0.01	0.27	0.46
B	1,690	0.31	0.57	0.65	4.91	0.21	0.39	0.47	0.52	0.08	0.04	0.39	0.48
C	1,602	0.49	0.66	0.84	4.98	0.21	0.46	0.51	0.43	0.14	0.12	0.24	0.58
D	1,198	0.42	0.67	0.82	5.04	0.28	0.42	0.46	0.49	0.21	0.42	0.38	0.74

CFE: Cell-free extract, CE: Culture extract, PG: Polygalacturonase PE: Pectin esterase, CMCase: Carboxymethyl cellulase

[a] Composition of media (g/*l*)

Medium A: Sucrose, 150 g; NH_4NO_3, 20 g; KH_2PO_4, 10g; $MgSO_4 \cdot 7H_2O$, 2.5 g, Medium B: Medium A + pectin 2 g, Medium C: Medium A + pectin 4 g, Medium D: orange peel 6% (W/W)

Units: Activity/mg protein

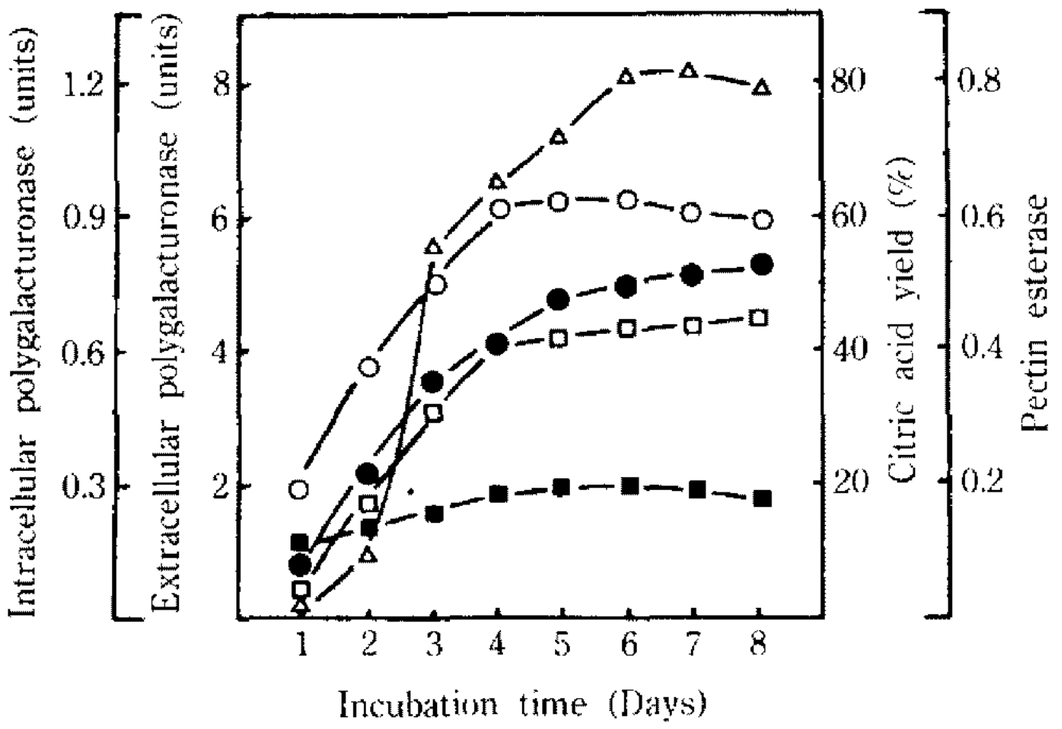

Fig. 1. Polygalacturonase and pectin esterase activities by *Asp. niger* in syntehtic medium.

○—○: Extracellular polygalacturonase
●—●: Intracellular polygalacturonase
△—△: Citric acid yield
□—□: Extracellular pectin esterase
■—■: Intracellular pectin esterase

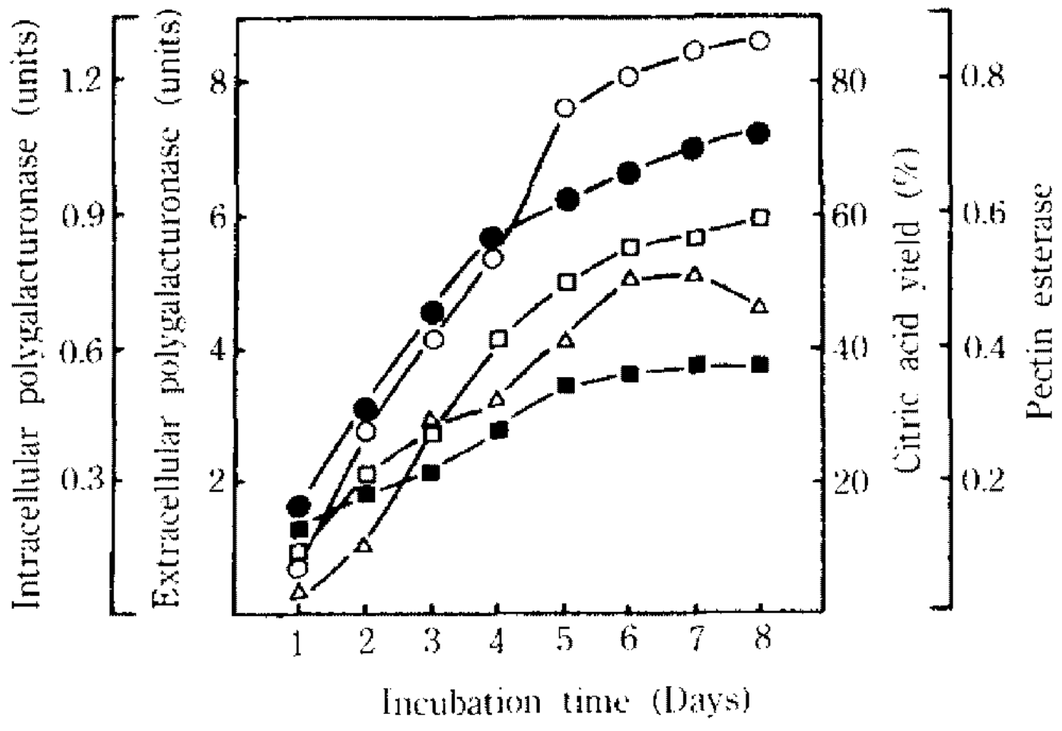

Fig. 2. Polygalacturonase and pectin esterase activities by *Asp. niger* in orange peel medium.

○—○: Extracellular polygalacturonase
●—●: Intracellular polygalacturonase
△—△: Citric acid yield
□—□: Extracellular pectin esterase
■—■: Intracellular pectin esterase

time on the activities of pectin digestable enzymes and citric acid yield. The activities of these enzymes higher in mandarin orange peel medium were than synthetic medium. As culture time increased, the activities of these enzymes and citric acid yield were increased in each medium. Therefore, there should be a close relationship between the activities of these enzymes and citric acid yield.

It is interesting to identify the mechanism that converts carbohydrate of mandarin orange peel to citric acid via glycolysis. Table 3 shows the results of comparison of key enzyme activities in EMP and HMP pathway. The activities of these enzymes and citric acid accumulation are increased, as incubation time increased. The activities of enzymes relating to EMP pathway were higher in synthetic medium than mandarin orange peel medium, and the activities to HMP pathway were *vice versa*. It suggested that HMP pathway was more active than EMP pathway in mandarin orange peel medium, and EMP pathway was more active than HMP pathway in synthetic medium. This result possibly indicates

Table 3. Change in the specific activities of enzyme of EMP and HMP during growth of *Asp. niger* under submerged culture

Enzymes	Synthetic medium Enzymes			Orange-peel medium Enzymes		
	Specific activity*			Specific activity*		
	3 days	5 days	7 days	3 days	5 days	7 days
EMP enzymes						
Hexokinase	0.42	0.84	0.65	0.38	0.64	0.69
6-phosphofructo Kinase	1.62	1.67	1.65	1.21	1.03	0.91
Pyruvate Kinase	3.24	5.37	7.42	3.62	5.32	7.78
HMP enzymes						
Glucose-6-phosphate dehydrogenase	1.30	1.06	1.03	1.18	1.14	1.12
6-Phosphogluconate dehydrogenase	0.67	0.43	0.38	0.69	0.54	0.48
Transketolase	0.06	0.06	0.12	0.12	0.34	0.21
Citric acid yield(%)	56.7	70.3	81.4	34.4	41.7	50.9

*: μmole/min./mg protein

Table 4. Change in the specific activities of enzymes of TCA cycle during growth of *Asp. niger* under submerged culture

Enzyme	Synthetic medium				Orange peel medium			
	Specific activity				Specific activity			
	2 days	4 days	6 days	10 days	2 days	4 days	6 days	10 days
Citrate Synthetase	0.06	0.42	0.75	0.65	0.06	0.41	0.70	0.69
Aconitase	0.21	0.03	0.03	0.24	0.19	0.03	0.03	0.25
I.D								
$NADP^+$-dependent	0.40	0.36	0.39	0.87	0.44	0.34	0.35	0.82
NAD^+-dependent	0.22	0.02	0.02	0.21	0.21	0.02	0.02	0.16

I.D: Isocitrate dehydrogenase, *: mole/min/mg protein

that is digested by pectin esterase as well as polygalacturonase. This result was also supported by the report of Church (16). As a result, it can be thought that citric acid is made from not only reducing sugars in mandarin orange peel *via* EMP pathway but also pectin and fiber substances in mandarin orange peel *via* HMP pathway, by various enzymes produced by *Aspergillus niger* GFM-0014.

There are a number of reports on citric acid accumulation *via* TCA cycle by *Aspergillus niger* under various alterations of fermentation condition, characteristics of each enzyme, and regulatory mechanism of metabolism (18). But there is no report on relation between the activities of enzymes relating to TCA cycle using mandarin orange peel as a substrate.

Principal enzymes relating to synthesis of citric acid in TCA cycle are citrate synthase, aconitase and NAD/NADP-dependent isocitrate dehydrogenase. Table 4 shows the result of activities of these enzymes. When the fermentation time was 6 days, the activity of citrate synthetase in synthetic medium was high. On the other hand, the activity of aconitase and NADP-dependent isocitrate dehydrogenase were slightly decreased. This aspect was not changed in mandarin orange peel medium.

요 약

만다린 오렌지 과피를 기질로 하여 *Asp. niger*의

구연산 발효를 행하여 관련된 일련의 효소적 활성을 합성배지와 비교한 결과 만다린 오렌지 과피배지에서는 과피에 함유된 Pectin이나 조섬유 등의 자화로 인하여 Polygalacturonase와 Pectin의 활성 뿐만 아니라 CMCase, xylannase 및 amylase의 활성이 높게 나타났다.

해당과정에 관련된 효소활성도는 합성배지에서는 EMP 경로에 관련되는 효소의 활성이 높은 반면 만다린 오렌지 과피 배지에서는 HMP 경로에 관여하는 효소의 활성이 높았다. 구연산 생산에 직접 관련된 citrate synthetase의 효소활성이 높을 때 구연산 생산이 증가되었으며 동시에 aconitase와 $NADP^+$-dependent isocitrate dehydrogenase의 활성은 낮게 나타났다.

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