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Influence of Carbohydrate and Nitrogen Source on Immunosuppressive Compounds Production by *Penicillium* spp.

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*Penicillium*菌에 의한 免疫抑制物質 生産에 있어서의 炭素源과 窒素源의 影響

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Abstract

Cultural conditions of shake-flask culture for the production of antimicrobial metabolites containing immunosuppressive activities by fungi isolated from peanut seeds were investigated. The thermotolerant stain, identified as *Penicillium citrinum* var Pa-6, produced maximum antimicrobial metabolites at 35℃ after 2 weeks on nitrogen-limiting medium. According to cultural conditions, optimum medium compositions for antimicrobial metabolites production were defind as follws; 1.5% of galactose with 0.5% dextrose, 0.046 % NH_4NO_3(C/N ratio 50), 10mM of Pi and trace elements. The expression of antimicrobial actvities were appeared sooner than control during cultivation with the addition of some amino acid such as phenylalanine, glutamine or tyrosine.

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Introduction

In the last ten years, a number of fungi have been reported to produce secondary metabolites capable of suppressing the immune system in laboratory animals and humans. one fungal metabolite, cyclosporin A, has recently gained worldwide attention as a potent immunosuppressive agent. The fungal metabolite gliotoxin has also been reported to have immunomodulatory activity(Mullbacher et al., 1985). The unknown compound which is not a cyclosporin A or gliotoxin, produced by the isolated stain Pa-6, has been reported to have immunosuppressive activity(Stack et al., 1988)

For over-prouduction of secondary metabolites, it is generally held that two principal ways can lead to improve production of microbial metabolites : (a) increase of biomass concentration which is usually limited by the availability of dissolved oxygen and (b) increase of cellular productivity due to manipulation of control mechanisms(Grafe, 1982).

In this study, we identified the stain Pa-6 isolated originally from peanut seed, and investigated cutural conditions of this strain for the over-production of antimicrobial metabolites with control of medium compositions, and defined medium compositions for shake-flask culture. The separation and identification of unknown compounds, mass production of this compounds with fermentor culture are in progress.

Materials and Methods

Strains : *Penicillium* spp. used throughout this study originally isolated from peanut seed(Stack et al., 1988). *Bacillus cereus* is used as a indicator strain in bioassay of antimicrobial activities. The strains were stored at room temperature and transfered every two weeks.

Media : Basal medium compositions in shake-flask culture are 2% maltose, 0.1% NH_4NO_3, 0.2% KH_2PO_4, 0.04% $MgSO_4.H_2O$, and 0.001% $FeSO_4.7H_2O$. Calcium carbonate (0.1%) was added for pH control and pellet formation of culture. The pH of the culture was adjusted to 6.0 by adding 1 N-NaOH solution before autoclaving. All other media were modifications of this. Stock culture and plate culture for bioassay are potato dextrose agar.

Culture : Shake-flask culture for nutritional requirements studies was carried out at 35℃ on rotary shaker at 130rpm. A small piece of slant culture was used to inoculate medium(30ml) in Erlenmeyer flasks of capacity 125ml. Inoculum size was about 8×10^5 spores/flask.

Bioassay : Antimicrobial activities of

culture filtrate were measured according to the method of William(1977) as follows. After cultivation, culture was filtered with membrane filter(pore size 0.45 μm). Twenty $\mu\ell$ of culture filtrate was injected into filter paper disc(0.65mm diam, Schleicher & Schuell #740-E), put it on the plate culture inoculated with *Bacillus cereus* previously, and then incubated at 35℃ overnight. Antimicrobial activity is expressed in arbitary units defined as follows. One unit is one mm of inhibition zone formed by culture filtrate of 20$\mu\ell$.

Analytical methods : After cultivation in indicated period, thirty ml of culture was fitered with VWR filter paper(#610). Dry cell weight was measured by drying the filter residues of the culture at 70℃ for one day. Carbohydrate contents in culture broth were determined by the method of Nelson(1944).

Identification of the stain Pa-6 : Identification of the stain was followed by the methods of Pitt(1979), Raper et al.(1949) and Bilai(1963).

Results

Identification of the strain Pa-6

Cultural characteristics : CYA, 25℃, 7days : Colonies 25-29mm diam, plane to slightly radially sulcate, sometimes convolute, growth low, moderately dense, velutinous to floccose, at times more or less zonate; mycelium white; conidiogenesis light, greyish green; exudate present, clear to pale yellow; soluble pigment bright yellow slightly present; reverse pale brown.

MEA, 25℃, 7days : Colonies 14-19mm diam, plane, centrally convolute, growth low and relatively sparse, strictly velutinous; margins narrow; conidiogenesis moderate, dark green; exudate and soluble pigment absent; reverse dull green.

G25N, 25℃, 7days : Colonies 12-15mm diam, plane, centrally convolute, dense, zonate, at centers pale yellow; mycelium white; conidiogenesis light, in colors similiar to those on CYA; exudate and soluble pigment absent; reverse pale yellow.

5℃, 7days : No germination.

35℃, 7days : Colonies 12-13mm diam, strictly sulcate; exudate pale yellow abundantly produced.

Conidiophores : Conidiophores borne from surface hyphae, stripes 93-290 x 2.4-3.6 μm, smooth walled, terminating in penicilli varying on CYA from bimetuate to verticils of 4 terminal and subterminal metulae, or occasionally ramulate, but on MEA rarely more complex than verticils of 2-3 metulae, and frequently monoverticillate; rami divergent, irregularly disposed, 21-43 x 2.4-2.9μm; metulae 7.1-

23.8 x 1.9-3.5μm, sometimes longer if solitary and subterminal; phialides in verticils of 3-5, ampulliform, 7.1-11.9 x 1.2-2.0 μm; conidia spheroidal to subspheroidal, commonly 2.5-2.9μm; smooth walled, borne on CYA in disordered chains, but MEA sometimes in long columns.

Distinguishing characteristics: The strain produces relatively long divergent metulae often of unequal length; smooth conidia borne on CYA in disordered chains; velutinous, low and spreading colonies on MEA. On MEA growth is usually slow and dense, with heavy conidiogenesis. Colonies on CYA at 35℃ are often dominated by copious clear to pale yellow exudate.

According to cultural and morphological characteristics, the strain is between *Penicillium citrinum* and *P. janthinellum*, but not fitted to any strain described in Pitt's "The genus *Penicillium*". This strain is very similiar

Table 1. Carbon source effect on the production of antimicrobial metabolites with cultivation time

Carbon surce	Antimicrobial activity(unit) *						Dry cell** (g/ℓ)
	5 day	7 day	10 day	13 day	15 day	18 day	
Dextrose	t***	t	0.5	1.0	7.5	8.0	5.84
Maltose	4.3	6.8	7.3	7.3	7.8	8.3	6.56
galactose	t	t	5.5	10.5	12.5	13.0	6.24
Sucrose	3.4	5.1	4.9	6.8	8.4	8.6	6.84
Starch(potato)	t	4.9	7.6	11.5	12.3	11.8	7.18
Glycerol	5.8	6.6	6.7	11.3	12.0	12.5	5.74
Lactose	4.3	7.7	10.7	10.9	12.7	11.5	3.27
Mannitol	t	3.3	6.0	6.5	8.3	7.0	5.60
Sodium citrate	t	t	0.7	0.7	0.5	0.5	2.73
Sodium fumarate	t	t	0.7	0.7	2.6	2.9	2.36
Vegetable oil	t	t	t	t	0.5	0.5	13.13

*One unit is defined as one mm of inhibition zone formed when 20 μℓ of culture filtrate was injected into filter paper disc(6.5mm diam) on potato dextrose agar plate inoculated with *Bacillus cereus* and incubated at 35℃ overnight.

**Afte cultivation as indicated period, dry cell weight was measured by drying the filter residue of whole culture(30ml medium/124ml flask).

***it is trace in inhibition zone formed.

to *Penicillium citrinum* on cultrual characters except on MEA, but on morphological characters is similiar to *Penicillium janthinellum* except that metulae are predominantly borne terminally. Therefore, this strain may be a variant of Citrina series of *Penicillium*. This strain is identified as *Penicillium citrinum* var Pa-6.

Nutritional requirements of antimicrobial metabolites production by this strain

The effect of carbon sources in medium compositions with shake-flask culture: Various carbon sources in medium compositions were investigated for the production of antimicrobial metabolites with cultivation time, and the results are shown in Table 1. Mono and disaccharides, espicially dextrose, maltose and sucrose, were good carbon sources for cell growth in initial stages, but higher antimicrobial activities of culture filtrate were shown in other carbon sources such as galactose, glycerol, lactose and starch. With the cultivation time, the antimicrobial activities of culture filtrate were increased gradually, and maximum activities were obtained after 2 weeks. Cultivation time for the expression of antimicrobial activities in dextrose, maltose and sucrose as a carbon source was prolonged compared to other carbon sources.

High viscous and dense culture was obtained on vegetable oil as a carbon source, but antimicrobial activity of culture filtrate was very low. Cell growth on sodium citrate or sodium fumarate was strongly inhibited with alkaline pH of cultrue.

The effect of nitrogen source in medium compositions with shakeflask culture: Table 2 shows the nitrogen source effect for the expression of antimicrobial activities with cultivation time. Carbon and nitrogen source were 2% maltose and 0.1% NH_4NO_3 respectively. Compared to Table 1, the expressions of antimicrobial activities were appreared sooner and higher for some amino acids such as phenylanine, glutamine, and tyrosine in initial stages of cell growth. Asparagine was a good source of nitrogen for this strain, but antimicrobial activity was not appeared during cultivation. Antibiotic synthesis can be suppressed by ammonia and other rapidly utilized nitrogen source(Aharonowitz, 1980), but there was not a difference on the expression of antimicrobial activities between ammonium salts and other nitrogen source when nitrogen was limiting in the culture medium as shown in Table 2. High nitrogen concentration in medium composition was good for cell growth, but antimicorbial activities were very low and were expressed very slowly in the culture. This results also show that antimicrobial

Table 2. Nitrogen source effect on the procuction of antimicrobial metabolites with cultivation time*

Nitrogen source	Antimicrobial activity (unit)						Dry cell** (g/ℓ)
	5 day	7 day	10 day	13 day	15 day	18 day	
NH_4NO_3	4.3	6.8	7.3	7.3	7.8	8.3	6.56
$(NH_4)_2SO_4$	t	4.0	6.8	9.0	8.8	9.2	5.49
$NaNO_3$	3.9	7.3	8.0	8.5	9.6	9.5	5.69
Alanine	5.3	5.8	7.3	8.5	10.2	8.8	6.30
Arginine	t	t	t	3.3	4.2	4.3	6.48
Asparagine	t	t	t	t	t	0.5	6.90
Cystine	0.5	4.3	6.3	6.3	6.5	3.2	4.17
Glutamine	6.0	7.0	9.0	8.3	8.7	7.3	7.00
Phenylalanine	6.8	7.2	8.8	8.3	9.0	9.3	3.87
Threonine	1.3	3.5	6.5	8.8	10.8	9.7	6.50
Tyrosine	6.0	7.6	7.6	8.8	9.0	10.4	3.85
Valine	3.9	4.9	6.0	9.3	9.3	9.4	4.38
Casein	2.2	7.5	7.8	8.5	8.8	9.8	6.60
Peptone	2.3	4.8	8.5	9.3	10.5	9.7	5.82

*Culture medium contained 2% maltose and 0.1% nitrogen source.

metabolites production was strongly suppressed by the presence of excess ammonia, furthermore, nitrogen regulation in medium compositions will be needed.

The effect of Carbon/Nitrogen ratio in medium compositions with shake-flask culture: Carbon/Nitrogen ratio in the medium compositions were varied from 10 to 100, and the strain was cultivated with shake-flask culture. As shown in Fig. 1. the expression of antimicrobial activities were appeared when nitrogen were used, higher activities were produced in same cultivation time compared to Table 1 and Table 2.

Higher C/N ratio than 70 in the medium compositions reduced the cell mass, and the culture broth colored to dark brown. Antimicrobial activities of culture filtrated were decreased slightly when cell growth was ceased.

The effect of phosphate amount in medium compositions with shake-flask culture: Phosphate is the crucial growth-limiting nutrient in many secondary metabolite fermentations; it is usually ex-

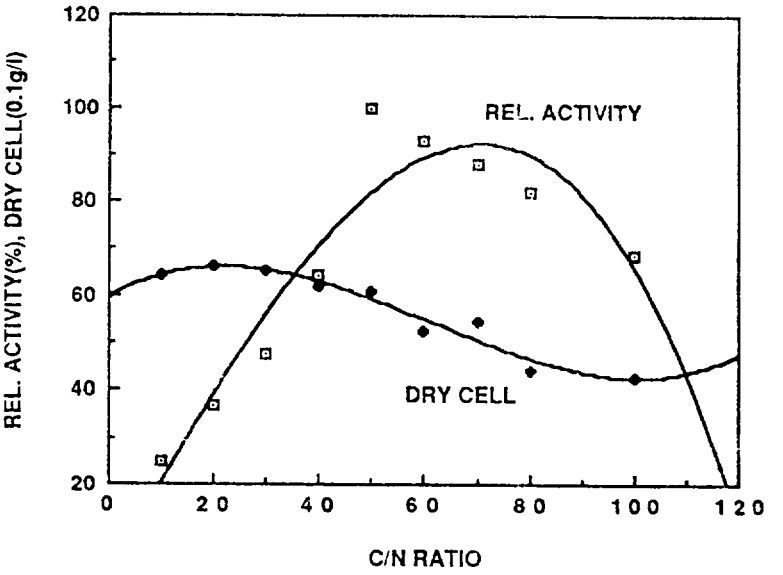

Fig. 1. Carbon/Nitrogen ration effect on the production antimicrobial metabolites.

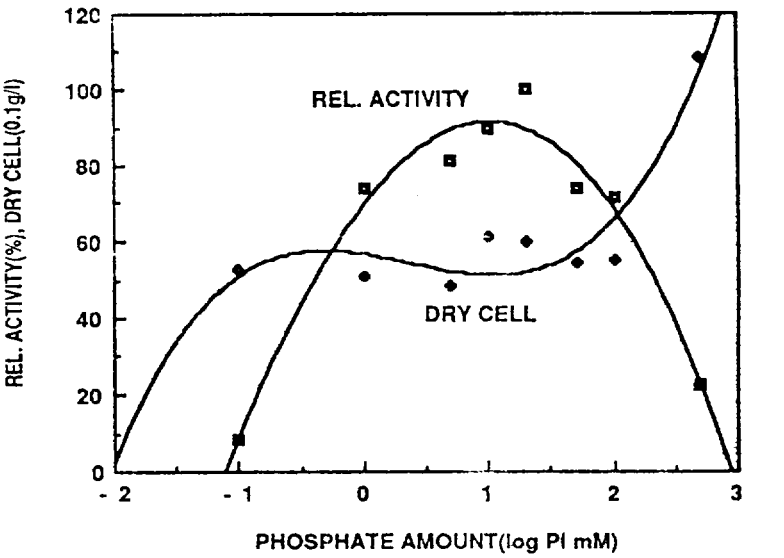

Fig. 2. Phosphate amount effect on the production of antimicrobial metatolites.

Table 3. Cosubstrate addition effects on the production of antimicrobial metabolits*

Substrate	Antimicrobial activity(unit)				Dry cell (g/ℓ)
	7 day	10 day	13 day	15 day	
Maltose	8.0	9.8	9.0	8.3	7.05
Galactose + Dextrose	9.6	11.4	11.8	11.8	6.73
Galactose + Veg. oil	7.9	10.8	7.8	8.2	14.66
Lactose + Dextrose	7.3	8.8	8.7	7.9	5.74
Maltose + Dextrose	6.4	9.7	11.2	11.3	6.56
Starch + Dextrose	4.6	9.2	10.9	10.2	6.91
Glycerol + Dextrose	9.3	11.1	11.4	12.0	6.32

*Carbon sources were consisted of 1.5% indicated carbon source and 0.5% dextrose. C/N ratio and phosphate were 50 and 20mM respectively. Trace elements consisted of 0.004% Mn $SO_4 \cdot 4H_2O$, $CusO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ respectively were added to the medium.

hausted during growth before the onset of product synthesis. It had been established that although vegetative growth is permitted throughout the range of 0.1 to 500mM Pi, secondary metabolic processes generally are restricted log concentrations of 10mM(Weinberg, 1978). Optimum Pi concentration of this strain was 10 to 20mM on the expression of antimicrobial activities as shown in Fig. 2.

Cosubstrate and amino acid addition effect on the expression of antimicrobial activities: As shown in Table 1, the production of antimicrobial metabolites was good on some carbon sources such as galactose, maltose, lactose, starch and glycerol, however, cell growth and the expression of antimicrobial activities were poor in initial stages of cultivation. Mutisubstrates which contains a substrate assimilating easily for cell growth and another one assimilating slowly for the production of antimicrobial metabolites would be needed for over-production as indicated in this experiments. Table 3 shows the cosubstrate addition effects on the expression of antimicrobial activities. By the addition of dextrose and trace elements, the expression of antimicrobial activities was appeared sooner and higher, compared to Table 1.

When this strain was cultivated with the addition of 0.01% selected some amino acid as shown in Table 2, the expression

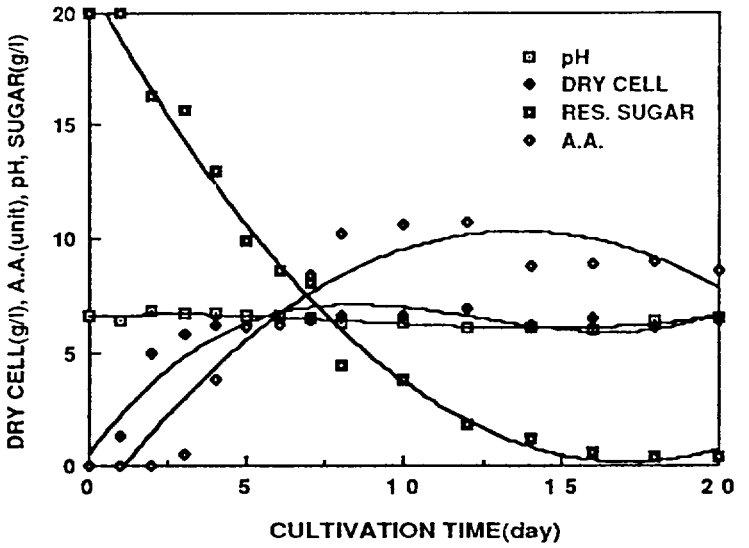

Fig. 3. Relation between cell growth, sugar consumption and the expression of antimicrobial activities on optimum cultural conditions

of antimicrobial activities were appeared sooner than control in initial stages of cell growth, but maximum antimicrobial activity and dry cell weights were not changed.

Relation between cell growth, sugar comsumption, pH changes and the expression of antimicrobial activities on optimum conditions is shown Fig. 3. Figure 3 shows a good relationship between cell mass and sugar consumption. The expression of antimicrobial activities was appeared after 3 days, and maximum antimicrobial activity was obtained in 10 days cultivation. The pH change of culture broth was decreased slightly during cultivation.

Discussion

In this study, we investigated the physiology of the isolated strain and cultural conditions for antimicrobial metabolite production. Secondary metabolic products are not produced when growth ceases, but that timing is a fuction of the nutritional state. The expression of antimicrobial activities was appeared gradually higher according to cell growth, but decreased slightly when growth ceased as shown in Fig. 3. This strain could assimilated sugar alcohols, fatty resources and organic acid as well as usual carbohydrates, but high antimicrobial activities were appeared on the medium containing carbon source assimilated slowly. Large and dense pellets were formed in rich medium compositions during cultivation, while relatively small

dense pellets were arised in poor cultural conditions.

The chemical composition of typical dry cells is approximately 40% carbon, 10% nitrogen, 1% sulphur, and 10% ash, with the remainder being oxygen(Calam, 1979). For nitrogen regulation of the cultrue in the secondary metabolites production, Carbon/Nitrogen ratio in the medium composition would be more than 4 theoritically. High C/N ratio contained inorganic nitrogen source or the organic nitrogen source such as amino acids and proteins released and assimilated slowly are good antimicrobial metabolites production as shown in Table 2 and Fig. 1.

This also suggests that the balance of carbon and nitrogen is important in regulating antimicrobial metabolites production. The ammonia effect is expressed early during the growth phase and thus enzymes(Aharonowitz, 1983). Excess ammonia in the culture medium contributed to cell growth, but suppressed strongly the production of antimicrobial metabolites in this study.

Whether antimicrobial activity would be derived from immunosuppressive compound produced by the strain in this study was not determined. However, there was a good correlation between antimicrobial activities and crude extract of culture filtrate in inhibition zone formed. This study would provide rapid and simple method in the determination of defined medium composition and physiology of the strain isolated originally for mass production of secondary metabolites.

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國 文 抄 錄

땅콩에서 分離한 곰팡이를 사용하여 免疫抑制活性을 갖는 抗菌性代謝産物의 生産을 위한 振湯培養條件을 檢討하였다. 耐熱性인 이 菌을 *Penicillium citrinum* var Pa-6으로 同定하였으며, 窒素源을 制限한 培地에서 35℃, 2주간 培養에서 最大의 抗菌作用을 나타냈다. 最適培地組成을 檢討한 結果, 1.5% 칼락토스와 0.5% 포도당을 炭素源으로 하여 C/N비율이 50이 되도록 0.046% NH_4NO_3를 窒素源으로 添加하고, 10mM 無機磷酸과 無機鹽類를 少量 첨가한 培地였다. 또한 페닐알라닌, 글루타민 또는 타이로신과 같은 몇 종류의 아미노산을 添加함으로써 抗菌作用은 培養중에 빨리 나타남을 알 수 있었다.