KOR. JOUR. MICROBIOL. June, 1990 p. 162-168 Vol. 28, No. 2

Effects of Culture Environments on Alkaline Protease Biosynthesis in *Streptomyces* sp.

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*Streptomyces*속 세균에서 호염기성 단백질 분해효소 생합성에 미치는 배양환경의 영향

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ABSTRACT: The aims of the present study were to evaluate the effects of culture conditions on the biosynthesis of extra-cellular alkaline protease in *Streptomyces* sp. The formation of aerial mycelia and spores were compared with the protease production in order to know the relations between the alkaline protease and the cell differentiation. As results, it was found that substrate concentration was very critical to regulate the formation of the protease, aerial mycelia, and spores, which were resulted from the changes of culture pH to acid. When the culture pH was adjusted with phosphate buffer from pH 6 to pH 9, the alkaline protease production was increased as the culture pH increased whereas aerial mycelia and spore formation were reversely related to the culture pH. Therefore, it was thought that the culture pH was an important factor to regulate the alkaline protease synthesis.

KEY WORDS□*Streptomyces* sp., Alkaline protease, Sporulation, Cell differentiation

Gram-positive, obligately aerobic *Streptomyces* bacteria are very interesting due to the formation of branching mycelia and spores. They form substrate mycelia of branching hyphae that can penetrate the interstices of the solid substrates. The coherent mycelial mass posseses highly concentrated hydrolytic enzymes located omni-presently at the growing tip of mycelia and the nutrients uptaken at advancing hyphal tips are transmitted inside the hyphae back to nutrient-limited regions. In response to nutrient limitation, the mycelia are turned endogeneously over to spores which are attached to the end of aerial hyphae (Hopwood *et al.*, 1970; Kendrick and Wheelis, 1982; Chater, 1984; Dworkin, M., 1985; Ensign, 1978).

It was reported that many genes were involved in the differentiation of *Streptomyces, viz.* the formation of branched mycelia and spores. Strains those were unable to form hairy spores were designated as bald mutants and *bld* loci regulating the spore formation were also described (Merrick, 1976). *bld A* mutant was reported to be a conditional mutant which could form spores on the media containing mannitol but not to form spores on glucose. However *bld B* mutant was unable to sporulate at any conditions, therefore, it was thought that an early stage of the processes was arrested (Babcock and Kendrick, 1988). Piret and Chater (1985) cloned *bld A* gene and the genetic complementation was also analyzed. And Lawlor *et al.* (1987) reported that the gene product of *bld A* resembled leucyl-t RNA molecule, but *bld B* gene product was not identified so far.

In other bacterial genus *Bacillus,* the spore formation was also linked to the formation of the extra-

cellular protease. Hyperproduction of neutral and alkaline proteases were phenotype of mutation at the *hpr* gene which encodes a negative regulator of protease production. The regulator could concomitantly control the genes required for sporulation (Perego and Hoch, 1988).

In previous studies, we found also that *Streptomyces* sp. SMF301 produced two different proteases of which otpimal pH were neutral and alkaline (Jeong *et al.*, 1988A). Non-sporulating mutants obtained from the treatment of acriflavin showed pleiotrophical changes in the production of alkaline protease and the formation of aerial mycelia. It was thought, therefore, that the cell differentiation and alkaline protease biosynthesis might be controlled closely with a certain regulatory mechanism (Jeong *et al.*, 1988B). However the molecular mechanism regulating on the both of sporulation and alkaline protease production has not been well understood.

In this context, it was contemplated to evaluate the effects of environmental factors which may affect on the biosynthesis of alkaline protease in conjunction with the cell differentiation in *Streptomyces* sp. SMF301.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Streptomyces* sp. SMF301 isolated from soil in Korea. Bald mutants (SMF302, 303) obtained from the parent strain after mutagenesis with acriflavin and NTG were compared. The characters were reported in elsewhere (Shin and Lee, 1986; Jeong *et al.*, 1988A). The strains were maintained by transferring to fresh stock culture media each week and storing at 10 °C.

Media

Stock culture medium was prepared with 0.3% of beef extract, 0.5% of peptone, and 1.5% of agar and seed culture medium was same to the stock culture except agar. Main culture medium was prepared with 2% of glucose, 1% of skim milk, 0.2% yeast extract, 0.1% of KH_2PO_4, 0.34% of K_2HPO_4, 0.01% of $MnCl_2 \cdot 6H_2O$, 0.01% of $MgSO_4 \cdot 7H_2O$, 0.025% of $FeCl_2 \cdot 2H_2O$, and 0.03% of Na_2CO_3. pH of the media were adjusted to 7.2 before steam sterilization.

Culture conditions

A single colony of *Streptomyces* sp. SMF301 developed on the plate of the stock culture medium was transferred to 100 m*l* of the seed culture medium and incubated in a rotary shaking incubator (150 rpm) for 2 days at 30 °C. The seed culture broth was inoculated into 1 liter of the main culture medium contained in the 2 liters culture vessel of fermentor (Korea Fermentor Co.). Temperature was maintained at 30 °C. Aeration and agitation were controlled to 0.5 vvm and 200 rpm, respectively.

Analytical methods

Activities of extracellular protease produced in solid cultures were compared with the diameter of clear zones which were resulted from the hydrolysis of casein contained in the main culture media. The activities of protease in submerged cultures were determined with the method reported previousely (Jeong *et al.*, 1988A).

The growth of cell on the main cultures was estimated by measuring the volume of packed mycelia (PMV) after centrifugation of the culture broth at $1000 \times g$ for 15 min. Quantitative analysis of the spore formation was carried out by counting as the followed procedures: 10 m*l* of the culture broth was homogenized by gentle agitation and 10 m*l* of 0.1 N HCl solution was added to the homogenized cell suspension. The mixtures were allowed at 30 °C for 10 min and cells were collected by centrifugation ($15,000 \times g$, 20 min). The cells were resuspended in the broth of the fresh seed culture medium and then the diluted cell suspensions were plated on the stock culture medium. Colonies developed after incubation at 30 °C for 3 days were counted and expressed as spore forming unit (SFU) (McBride *et al.*, 1987A; 1987B).

RESULTS AND DISCUSSION

Effects of medium composition on the formation of alkaline protease and cell differentiation

In order to know the effect of medium composition on the cell differentiation and alkaline protease production of *Streptomyces* spp., the strain was inoculated into the seed culture and the main culture medium individually and cultivated at 30 °C for 5 days, then the cell growth and alkaline protease production were compared. As shown in Table 1 and Fig. 1, it was clear that alkaline protease activity in the culture broth using stock culture medium was very much lower than that of the main culture medium. Furthermore it was very interesting to note that the mycelia formed in the seed culture medium were straight, whereas the mycelia grown in the main culture medium were highly branched. In addition, spores bearing on the aerial mycelia were found only from the main culture medium but not from the seed culture medium.

It was thought that more protease should be pro-

Table 1. *Effect of culture media composition on the formations of alkaline protease production and cell differentiation in Streptomyces sp. SMF301*[a]

Medium	Composition	Initial pH	Final pH	Alkaline protease activity (units/ml)	Cell Differentiation
Seed culture medium	Beef ext. 0.3% Peptone 0.5%	7.0	9.0	1.6	Fig. 1A
Main culture medium	Glucose 2.0% Skim milk 1.0% Yeast ext. 0.2% KH_2PO_4 0.1% K_2HPO_4 0.34% Trace minerals[b]	7.0	8.4	253.8	Fig. 1B

[a]The culture was carried out at 30°C for 5 days.
[b]$MnCl_2 \cdot 6H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01%, $FeCl_2 \cdot 2H_2O$ 0.025%, and Na_2CO_3 0.03%.

duced in the main culture medium where skim milk was added as a major nitrogen source, since extracellular protease was necessary for the utilization of the protein. However it was not clear why the cells were differentiated only in the main culture medium but not in the seed culture medium. The major difference between the seed culture medium and the main culture medium was the ratio of carbon source to nitrogen source (C/N).

In order to know the effects of carbon and energy source on the cell differentiation of *Streptomyces* sp., wild strains (SMF301, SMF301-1), and bald mutants (SMF302, SMF303) were inoculated on the main culture agar medium where different carbon and energy sources were used. Then the plates were incubated at 30°C for 5 days and the production of extra-cellular protease and spores were compared. As shown Fig. 2, it was evident that the wild strains (SMF301, SMF301-1) produced more spores and extra-cellular protease at any conditions compared with the mutants (SMF302, SMF303). The spore formation and protease production of the mutants were apparently repressed at the media containing glucose, starch, cellobiose, and arabinose, but were not significantly repressed at the media containing fructose and lactose. Therefore it was thought that the mutants were classified as *bld A* (Babcock and Kendrick, 1988).

It was found also that the spore formation and extra-cellular protease production of the wild strains were apparently higher at the media containing glucose compared to starch. Hence, it was necessary to evaluate the effects of initial glucose concentration on the sporulation and alkaline protease production.

As shown in Table 2, it was clear that the initial concentration of glucose was very critical for alkaline

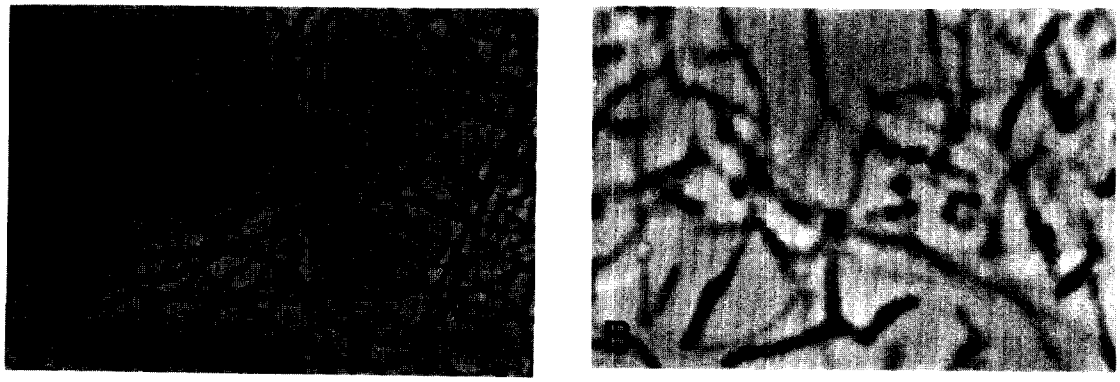

Fig. 1. *Microphotographs of vegetative mycelia and submerged spores of Streptomyces sp.* SMF301 grown at the seed culture medium (A) and the main culture medium (B).

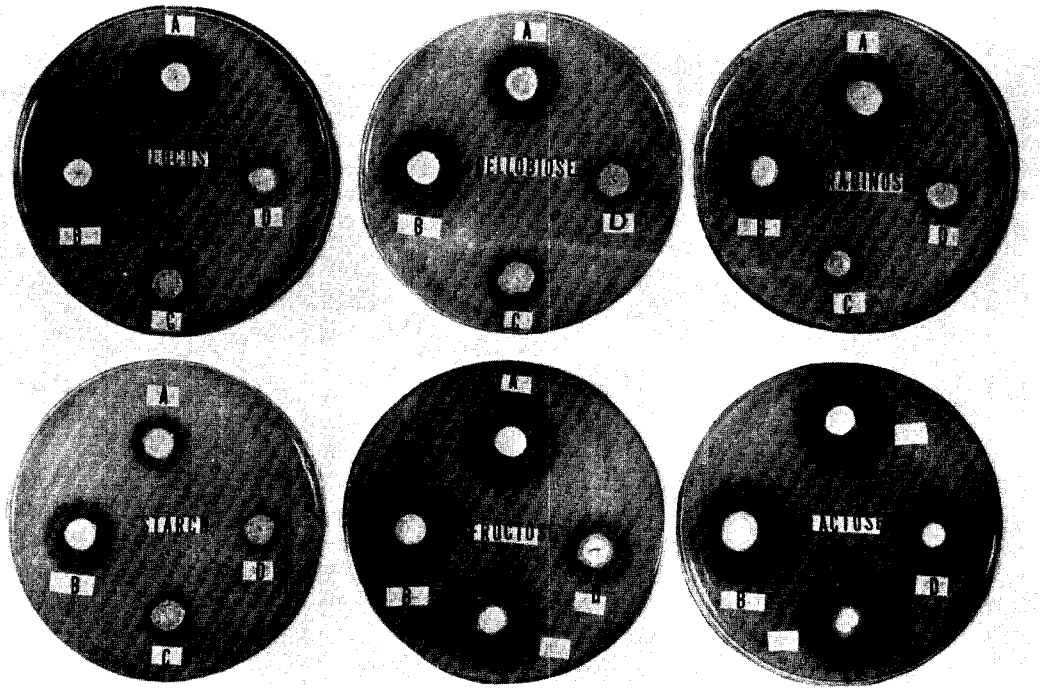

Fig. 2. *Effects of carbon and energy source on the production of alkaline protease and spore formation in wild strains (A and B) and bald mutonts (C and D) of Streptomyces sp. SMF 301*

protease production. Microscopic observation showed that the mycelia in the culture containing no substrate were straight but not branched greatly and that aerial mycelia and spores were not detected (Data are not shown here). The results were similar to those obtained from the culture using stock culture medium where substrate was also not added (Fig. 1A). It was thought that the concentration of substrate in the culture was important to regulate the cell differentiation. The optimal initial concentration of glucose for the production of spores, aerial mycelia, and alkaline protease was found to be 2%. And further increases in the initial glucose concentration might decrease the pH values. These results indicated that substrate concentration was critical for the optimal cell differentiation and also that some metabolites produced when substrate was excessive might give rise to decrease the pH of culture broth, which in turn, to repress the enzyme synthesis.

Enzyme level at Different culture pH

It was necessary to elucidate the effects of culture pH on the enzyme synthesis at medium containing high concentration of glucose, hence, the culture pH was adjusted by addition $CaCO_3$ or phosphate which could prevent rapid changes of culture pH. As shown in Table 3, it was clear that the growth of cell, spore formation, and the biosynthesis of alkaline protease were very much enhanced by adjusting the culture

Table 2. *Effect of glucose on the cell growth, alkaline protease production, and spore formation in the submerged cultures of Streptomyces sp. SMF301*

Carbon source	Conc.	pH	Cell growth (PMV, %)	Alkaline protease activity (units/ml)	Spore forming unit (ml^{-1})
Glucose	0%	9.8	3.9	37	1.8×10^5
	2%	8.7	7.0	437	1.6×10^7
	4%	4.8	3.1	63	3.8×10^6
	6%	4.6	1.5	26	3.0×10^5

*The culture was carried out using the main culture medium at 30°C for 5 days.

Table 3. *Effect of glucose on the cell growth, alkaline protease production, and spore formation in the buffered submerged cultures of Streptomyces sp. SMF301*

Buffered medium	pH	Cell growth (PMV, %)	Alkaline protease activity (units/ml)	Spore forming unit (units/ml)
Glucose 6%				
no buffered	4.5	1.1	94	2.5×10^5
0.5% $CaCO_3$	6.0	7.0	466	1.0×10^7
0.1M phosphate buffer	7.2	8.0	317	1.0×10^6

*The culture was carried out using the main culture medium at 30°C for 5 days.

pH. Therefore it was thought that culture pH played a very important role in the synthesis of essential enzymes for the growth of *Streptomyces* spp.

The effect of initial culture pH on the cell growth, cell differentiation, and alkaline protease production were also evaluated and the data are listed in Table 4. It was clear that the culture pH values were biologically adjusted to neutral pH, although the initial pH values were either acid or alkaline. The production of alkaline protease was enhanced as increasing of the initial pH values. The cell growth was closely related also to the culture pH and the mode of growth was observed to be more tip growth rather than branching growth at higher culture pH, which could increase the cell mass expressed as packed mycelia volume (PMV). However, it was interesting to note that the spore formation was reversely related to the cell growth and the alkaline protease production.

The SDS-PAGE proteins pattern of cells grown different pH were compared (data are not show here). It was very clear that proteins were clearly separated to each others at the cells cultured at pH 6.0, however, no distinct protein bands were recognized from the culture grown at alkaline pH.

Effect of phosphate concentration

It was evident that the optimal pH for the production of alkaline protease was alkaline. However, Table 3 shows that the alkaline protease production in the culture buffering with phosphate was much lower than that in the culture buffering with $CaCO_3$, although the final pH at the culture with phosphate was alkaline compared to the culture with $CaCO_3$. In this context the effect of phosphate concentration on the protease production was evaluated and the data are shown in Table 5. It was clear that the optimum concentration of phosphate for the growth and alkaline protease production was 0.03M and also that the cell growth and spore formation were closely related to reduce as increasing phosphate concentration. The molecular mechanism of the inhibitory effects of phosphate was not well discussed in conjunction with the cell differentiation. However it was evident that the synthesis of secondary metabolites were apparently repressed at hight concentration of phosphate (over 20 mM) (Ensign, 1988).

Effect of nitrogen source

In order to know the effects of nitrogen source on the alkaline protease biosynthesis and spore formation of *Streptomyces* sp., wild strain (SMF301) was inoculated on the main culture medium culture where different nitrogen sources were used. Then the cultures were carried out at 30°C for 5 days and the pro-

Table 4. *Effect of initial culture pH on the cell growth, alkaline protease production, and spore formation in the submerged cultures of Streptomyces sp. SMF301 using phosphate buffer*

Initial pH	Final pH	Cell growth (PMV, %)	Alkaline protease activity (units/ml)	Spore forming unit (units/ml)
6.5	6.8	7.9	145	2.1×10^9
7.0	7.1	9.3	191	8.5×10^8
7.5	7.5	12.1	417	5.9×10^8
8.0	7.6	12.8	622	4.1×10^7
9.0	7.8	13.0	679	2.0×10^7

*The culture was carried out using the main culture medium at 30°C for 5 days.

Table 5. *Effect of inorganic phosphate on the cell growth, alkaline protease production, and spore formation in the submerged cultures of Streptomyces sp. SMF301*

Conc. of phosphate (M)	Final pH	Cell growth (PMV, %)	Alkaline protease activity (units/ml)	Spore forming unit (units/ml)
0	8.3	4.0	26	5.1×10^8
0.02	8.8	7.9	185	6.8×10^7
0.03	7.7	18.0	670	1.3×10^8
0.10	7.4	4.3	345	9.3×10^8
0.20	7.2	7.5	172	6.0×10^7
0.30	7.2	11.5	161	7.4×10^6
0.40	7.2	9.8	143	6.4×10^5

*The culture was carried out using the main culture medium at 30°C for 5 days.

duction of extracellular alkaline protease and spores were compared. As shown in Table 6, alkaline protease production in the culture containing arginine as a sole nitrogen source was slightly higher than that in the culture containing skim milk but alkaline protease production in the culture containing $(NH_4)_2HPO_4$ was much lower. Spore formation was the highest in the culture containing skim milk and the lowest in the culture containing $(NH_4)_2HPO_4$. These results indicated that readily utilizable nitrogen source repressed the synthesis of alkaline protease and that the production of alkaline protease and cell differentiation were closely related to the availability of nitrogen sources. It was also interesting to note that arginine is a useful nitrogen source for the construction of a chemically defined medium to produce alkaline protease.

Table 6. *Effect of nitrogen source on the cell growth, alkaline protease production, and spore formation in the submerged culture of Streptomyces sp. SMF301*

Nitrogen source	Conc.	Final pH	Cell growth (DCW, g/l)	Spore forming unit (units/ml)	Alkaline protease activity (units/ml)
$(NH_4)_2HPO_4$	(2 g/l)	6.59	5.38	4.3×10^7	74
arginine	(2 g/l)	6.95	5.35	1.1×10^8	285
skim milk	(10 g/l)	7.06	5.59	4.2×10^8	264

*Basal medium; Glucose 2%, $NaH_2PO_4\cdot2H_2O$ 0.468%, K_2HPO_4 0.52%, $MnCl_2\cdot6H_2O$ 0.01%, $MgSO_4\cdot7H_2O$ 0.02%, $FeCl_2\cdot2H_2O$ 0.025%, Na_2CO_3 0.03%.

**The culture was carried out at 30°C for 5 days.

적 요

토양에서 분리한 放線菌 一株에서 호염기성 단백질 분해효소의 생합성과 세포분화와의 관계를 규명하고자 기균사와 포자의 형성, 그리고 단백질 분해효소의 생산에 대한 배양조건의 영향을 조사하였다. 그 결과, 기질의 농도가 단백질 분해효소, 포자, 그리고 기균사의 형성의 조절에 매우 중요하며 이것은 배양액의 pH가 산성으로 변화되기 때문임을 알았다. 인산염 완충용액을 이용하여 배양액의 pH를 6으로부터 9로 조정하여 주었을 때 단백질 분해효소의 생성은 pH가 증가함에 따라 증가하였다. 이와 같은 결과로부터 배양액의 pH가 호염기성 단백질 분해효소 생합성의 조절에 중요한 요소로 작용한다고 판단하였다.

ACKNOWLEDGEMENT

This research was supported by a grant from the Ministry of Education through Genetic Engineering Research Program. The authors are grateful for the supports.

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(Received November 28, 1989)
(Accepted May 15, 1990)