재조합 *Pseudomonas Putida*의 내열성리파제 생산조건에 관한 연구

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Effects of Culture Conditions on the Production of Thermostable Lipase by Recombinant *Pseudomonas Putida*

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INTRODUCTION

An extracellular lipase produced from *P. fluorescence* SIK W1 has very high thermostability good enough to be used for the hydrolysis of beef tallow or palm oil which are not liquids at ambient temperatures. Recently, an effort was made to clone this thermostable lipase from *P. fluorescence* and to produce in recombinant *E.coli* (1). Induction of the tac promoter, which is in the upstream of inserted lipase gene with its apparent signal sequence and its own promoter, resulted in the formation of inclusion bodies in the cytoplasm. Refolding process of these inclusion bodies includes series of expensive and complicated steps which increase the production cost of the lipase. To produce lipase in a soluble form, extracellularly, we made an attempt to clone the lipase gene from *P. fluorescence* into multiple copy plasmid of *Pseudomonas* and transform into *P.putida* by electroporation (2). Few studies were reported on the recombinant lipase production by *Pseudomonas* species. In this study, culturing parameters influencing the production of recombinant lipase by *P.putida* were investigated.

MATERIALS AND METHODS

Plasmid construction and strain

As shown in Fig.1, a 1.6kb fragment of *P. fluorecence* lipase gene from the plasmid pJH92, was inserted into a multi-copy plasmid pDSK519. And the recombinant plasmid pDSK519-lip was introduced into the *P.putida* 1641 by the electroporation as described previously(2). Recombinant cells were kept at -70℃ with 25% glycerol after grown in the basal medium containing Bacto-peptone (2%), $MgSO_4$(0.05%), KH_2PO_4 (0.1%) and olive oil(1%).

Culture conditions

Frozen cells (0.5ml) were inoculated into 50ml basal medium in a 500ml Erlenmyer flask and cultivated for 10 days in a shaking incubator.

Fermentor culture was performed with 1 liter working volume in a 2.5L jar fermentor (KFC, Korea) at 26℃, 500rpm, with aeration rate of 1.5 vvm.

Assays

Lipase activity of the supernatant in the broth was determined by modified Cupric-Acetate method (3). One unit of the lipase corresponded to 1 μmol of free fatty acid released per minute.

RESULTS AND DISCUSSIONS

Plasmid stability and lipase activity

The plasmid stability of *P.putida* 1641 /pDSK519-lip was examined in a semi-continuous flask culture. As shown in Fig.2, the plasmid was generally stable over 25 generations. Among the tested *Pseudomonas* strains as host cells, recombinant *P.putida* 1641 showed the highest lipase activity. When the lipase activity of recombinant strain was compared with host strain, about 15 folds increase in the lipase activity was observed (Table 1). Measurment of lipase activity in the supernatant and whole cell prepared by centrifugation of stationary phase cells showed that over 95% of the total activity was present in the culture supernatant.

Effects of carbon and nitrogen sources

As shown in the Table 2, olive oil had a greater enhancing effect on lipase production than did other lipids, fatty acids and esters. When the concentration of olive oil was increased, the lipase activity decreased significantly, presumably indicating the suppression of enzyme production by high concentration of olive oil. Additions of IPTG (Isopropyl Thiogalactopyranoside), or lactose as inducers of lipase production were inefficient (data not shown). Among the tested nitrogen sources for the lipase production, 2% peptone as organic nitrogen and 1% $NaNO_3$ as inorganic nitrogen were found to be optimum conditions, respectively (data not shown).

Effect of surfactants

As is obvious in the Table 3, Tween-80 was effective in the lipase production among the various combinations of surfatants and olive oil. When tween 80 and olive oil were added equally to the medium, 30unit/ml of lipase activity was achieved. Surfactant such as Tweens or Spans were found to increase the lipase activity of *P.aeruginosa* (4).

Effect of pH , temperature and aeration.

Optimum pH and temperature for the lipase production were found to be 26 ℃ and pH7.0, respectively (data not shown). The lipase production of recombinant *P.putida* 1641 was unusally sensitive to cultivation temperature while optimum temperature of lipase production by other *Pseudomonas* species ranged from 23℃ to 37℃ or between 34-40℃(4,5). The effect of aeration on lipase production was investigated by changing the culture volume. Compared with culture of 150ml culture volme in 250ml flask, 5 folds increase in the activity was achieved in the well-aerated culture, i.e., 50ml culture volume in 250ml flask (data not shown).

Batch cultivation

Following the investigation of cultivating parameters on the lipase production in the flask culture, batch cultivation in the fermentor was performed with optimimum conditions. Under optimum conditions 60 (unit/ml) of lipase was produced in 48 hours.(data not shown).

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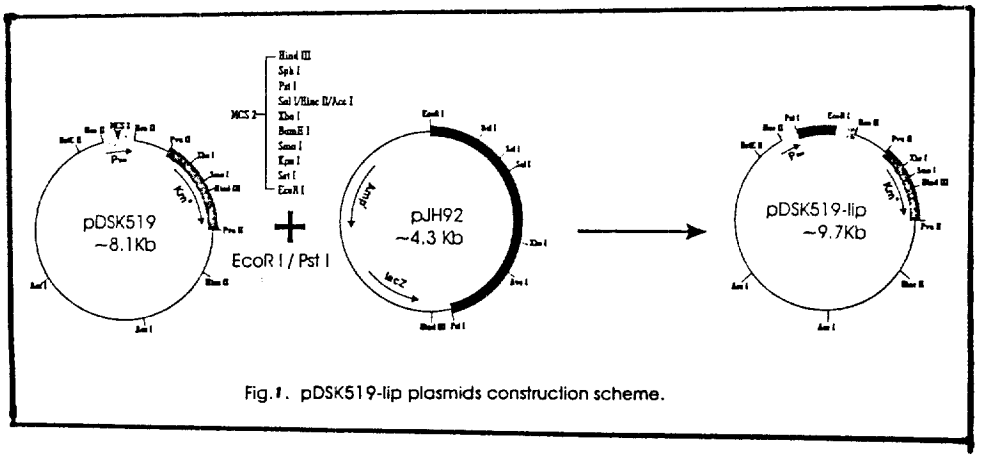

Fig.1. pDSK519-lip plasmids construction scheme.

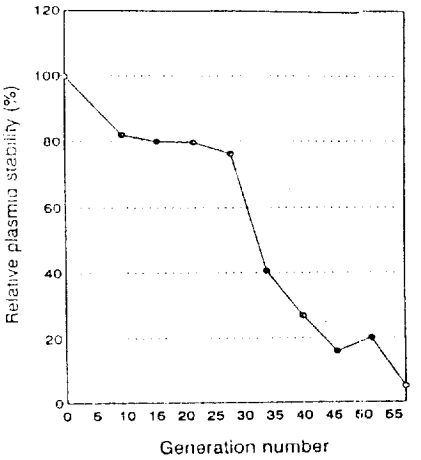

Fig.2 plasmid stability of
P. Putida 1641/pDSK519-lip

Table 1. Comparison of Lipase Activity

	Cell Growth O.D. $_{600nm}$	Extracellular Lipase Activity (U/ml)	Specific Activity (U/OD · ml)
P. fluorescence	12.0	0.88	0.07
P. putida 1641	13.5	1.60	0.12
P. putida/pDSK519-lip	13.4	20.20	1.51

Table 2. Effect of C-Source on Lipase Production

Property / C-source	Cell Growth O.D. $_{600nm}$	Extracellular Lipase Activity (U/ml)	Specific Activity (U/OD · ml)
Lipid			
Coconut oil	9.77	5.44	0.56
Peanut oil	9.86	4.92	0.50
Linseed oil	12.21	5.01	0.41
Olive oil 1%	7.74	20.20	2.61
3%	4.52	1.03	0.23
5%	3.70	1.08	0.29
Fatty_acid			
Palmitic	9.70	6.14	0.63
Linoleic	19.40	5.38	0.29
Oleic	16.60	5.35	0.32
Lauric	9.46	2.26	0.24
Myristic	8.10	0.52	0.06
Stearic	10.20	13.16	1.29
Ester			
Triolein	29.80	6.36	0.22
Glycerol	9.50	0.64	0.07
Glucose	11.50	0.15	0.013

Table 3. Effect of Surfactant on Recombinant Lipase Prodution

	Cell Growth O.D. $_{600nm}$	Extracellular Lipase Activity (U/ml)	Specific Activity (U/OD · ml)
Tween-80**	5.10	20.93	4.10
Span-80*	9.27	3.24	0.35
Olive Sp-80			
4:1	8.40	23.89	2.84
1:1	11.00	15.61	1.42
1:4	7.30	18.70	2.56
Olive:Tw-80			
4:1	9.00	9.35	1.04
3:2	11.50	13.85	1.20
1:1	9.50	30.08	2.62
2:3	6.40	25.58	4.00
1:4	9.20	24.44	2.66
Sp-80 Tw-80			
1:1	12.00	17.94	1.50

** Tween-80 : Tw-80 - Polyoxyethylene sorbitan monooleate
* Span-80 : Sp-80 - Sorbitan monooleate