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Studies on Intergeneric Protoplast Fusion and L-Lysine Productivity

In-Seon Lee*·Jung-Il Cho

**Dep. of Food Science and Technology, Keimyung University, Taegu*

Dep. of Agricultural Chemistry, Korea University, Seoul

Abstract

For the improvement of the L-lysine productivity of *Brevibacterium flavum and Corynebacterium glutamicum*, fusants were induced by interspecific protoplast fusion of *Bacillus subtilis* with *C. glutamicum* and B. flavum. The following results were obtained through protoplast formation of strains, condition of protoplast fusion, characteristics of the fusants, and the productivity of lysine from starch. *B. flavum* BF-5 and *C. glutamicum* protoplasts were made by the treatment of 0.3 unit/mℓ of penicillin G at the early stationary growth phase for 2 hours followed by incubation with 10mg/mℓ of lysozyme at 37℃ for 120 min. When a mixture of the protoplast was treated with 30% PEG(M.W.6,000) solution containing 50mM $CaCl_2$ at optimal conditions, the intergeneric fusion frequency between protoplasts of *C. glutamicum* CG-2 and B. subtilis BD 224 was 7.1×10^{-5}.

The genetic properties on the L-lysine producing fusants obtained were characterized, aspartokinase activity and L-lysine productivity of fusants were compared with those of parental strains. As a results, the intergeneric fusants were completed in each auxotrophic requirement, resistances for S-(2-aminoethyl)-L-cysteine and kanamycine were confirmed. And one of fusants selected, FBB-41 were found to be genetically stable fusants. The aspartokinase activity of FBB-41 strain increased than that of the parent strain.

Key word : Protoplast fusion·lysine production.

Introduction

The study on overproduction of essential amino acid with L-lysine has been made widely. The supplement of essential amino acid with lysine is needed to provide lysine which is lacked of for the people who live on grain and also to build up nutritional process in fodder industry. Moreover, the utilization of essential amino acid has been extended using it for spice and fortified food and also as nutrient and remedy in medical fields[1].

Previously, the amino acid production by microorganism was depend on the sorting method of bacteria which could produce specific amino acid from wild strains in natural world, but more amino acids have become produced by controlling the amount of enzyme or substrate on the process of biosynthesis of object metabolic material due to the utilization of reports and studies on meta-

bolism of microbes[2].

Many studies have been done recently to develop more economic bacterial strains and also to increase it's productivity and to reduce fermentation cost by the importation of genetic engeering technology. Cell fusion, gene transformation and gene recombinant have been known as a gene engineering technology, but the study of their application on *Brevibacterium* and *Corynebacterium* species which are good for producing lysine, has never been reported[3-9]. Kanasawa et al[10]. have developed excellent L-lysine producing bacterial strains through protoplast fusion using *Brevibacterium flavum* in 1986.

This study was carried out to examine the reducibility of production cost in fermentation industry of L-lysine, *Brevibacterium flavum* of homoserine auxotrophic mutant and *Corynebacterium glutamicum* of threonine auxotrophic mutant and for the intergeneric protoplast fusion between these bacteral strains *Bacillus subtilis* which has capability of amylosis was applied.

Materials and Methods

Bacterial strains

Parental strains and their fusants used in this experiment are shown on Table 1. Each of parental cells was purchased by American Type Culture Collection(ATCC).

Culture media

Luria-Betrant(LB) culture media was used as a complete culture media. As a basic culture media 20g glucose, 10g$(NH_4)_2SO_4$, 3g urea, 1g KH_2PO_4, 0.4g $MgSO_4 \cdot 7H_2O$, 0.05g NaCl, 0.02g $FeSO_4$, 200g biotin and 50g thiamine·HCl were dissolved in distilled water to be 1L of final volume. Required amino acid was adding by the concentration of 1 mg per 1L and used as culture media for the selection of fusant. According to Akamatsu et al[8], the complete culture media (CM) adding sodium succinate by 0.5M concentration and the minimal culture media with bovine serum albumin were used as protoplast regeneration culture media.

Isolation of auxotrophic mutant and AEC resistant mutant

Auxotrophic and AEC resistant mutant were isolated, and the resistances serve as convenient genetic selection markers for the protoplast fusion. The selection of mutant was followed by the report of Alderberg et al[12]. using N-methyl-N′-nitroguanidine(NTG) as a mutagen, the mu-

Table 1. List of strains used

strains	phenotype and genotype	source
B. flavum BF-5	Hse , Leu , AEC^r	NTG mutant of *B. flavum* ATCC 21474
C. glutamicum CG-2	Thr , AECr	NTG mutant of *C. gulutmicum* ATCC 14296
B. sutilis BS-7	Hse , Met , Km^r	Transformant of *B. subtilis* ATCC 21008
B. subtilis BD224	Trpc2, thr5, recE4	D. Dubnau

(Abbreviation).

Hse : Homoserine.
Leu : Leucine.
The : Threonine.
Met : Methionine.
AEC : S-2-(aminoethyl-L-cysteine).
Km : Kanamycine.
Trp : Tryptophan.
rec : recombination.

tant were concentrated with penicillin G and selected into two groups for nutrient requirement mutant by the method of replica and for AEC resistant to be separated on the basic culture media with 0.1% AEC.

Protoplast formation and cell wall regeneration

Each of bacterial cells was separated by centrifuge after cultivating by log phase and collected. Protoplast formation was induced by suspending these cells in SMM buffer added lysozyme by the concentration of 4mg per 1mℓ. Rate of protoplast formation was measured by the number of colony shown on the CM culture media covered with corresponding amount of osmotic shock treated protoplast inducing media. Regeneration of protoplast after cell fusion was carried out by the method of Akamatsu[8].

Result and Discussion

Isolation of mutants

To give genetic selection marker for the protoplast fusion on *B. flavum.*, *C. glutamicum* and *B. subtilis* used as parental cells before attempting intergeneric protoplast fusion, each of cells was treated by NTG and separated into nutrient requirement mutants and AEC resistance mutants. Finally, *B. flavum BF-5*, *C. glutamicum* CG-2 and *B. subtilis* BS-7 were selected by separation and *B. subtilis* BS-7 was used in this experiment after giving it kanamycin resistance additionally. These mutants showed the character which had longer term lag phase than wild bacterial strains before the treatment of mutation.

Protoplast formation and cell wall regeneration

According to the test of effects on protoplast formation in cell fusion, as shown on Fig. 1, most of cells formed protoplast in the 10mg/mℓ concentration of lysozyme for *B. subtilis* but protoplast formation was not done through simple treatment of lysozyme for *C. glutamicum* CG-2 and *B. flavum* BF-5. Considering that lysozyme does not affect on *C. glutamicum* CG-2 and *B. flavum* BF-5 during the activity of lysozyme affecting on protoplast formation which melts cell wall by cutting β-1, 4-glucosidic bond between the cell wall of constituent ingredients of gram-positive bacterium, N-acetyl glucosamine and N-acetyl murate of peptidoglycan layer, peptidoglycan layers of these bacteria seem to have different structures.

The study of protoplast formation managing penicillin G treated on proliferation. As shown in Fig. 2, the maximum protoplast formation rate was showed using lysozyme after treat penicillin G by 0.3unit/mℓ concentration on the log phase in the bacterial strains of *C. glutamicum* CG-2

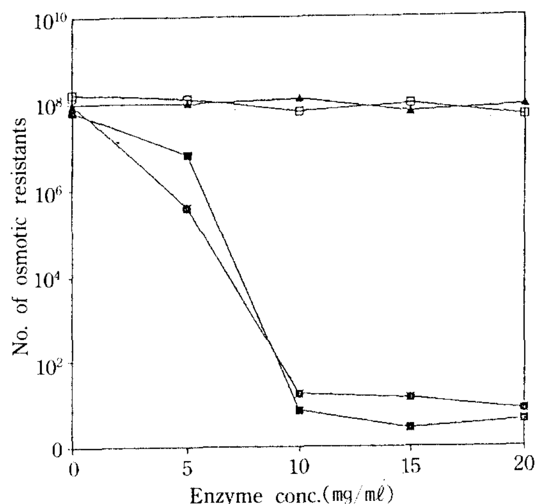

Fig. 1. Effect of lysozyme concentration on bacterial protoplast formation.
The cells were treated with lysozyme at 37℃ for 120min.
Symbols : ▲−▲, *C. glutamicum* CG-2 : □−□, *B. flavum* DF-5 : ■−■, *B. subtilis BD 224* : ●−●, *B. subtilis* BS-7.

and *B. flavum* BF-5. The cell wall regeneration that a formed protoplast returns to native cell was low when the treatment time was long and the concentration of lysozyme was high, and the regeneration rate off *C. glutamicum* CG-2 and *B. flavum* BF-5 in this experiment was about 10.2%, on the contrary, those of *B. subtilis* BS-7 and *B. subtilis* BD224 were 13.2% and 12.5%. Comparing this fact to the result of Kaneko and Sagakuchi[3], the rate is low but is relatively good in the comparison to the cell wall regeneration rates of *B. megaterium* of Foder[6]. The difference of regeneration rate in kinds of bacterium is considered to be caused by sensitivity of each bacterial strains to the lysozyme and the kind of lysozyme used.

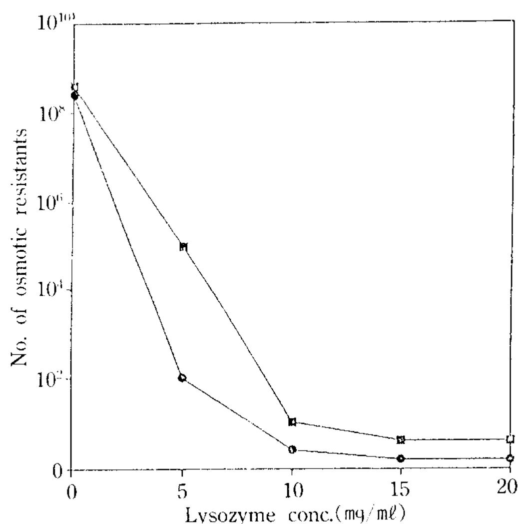

Fig. 2. Effect of lysozyme concentration on protoplast formation of penicillin G treated cell.
The cells were treated with lysozyme at 37℃ for 120min.
■－■, *Brevibacterium flavum* BF-5 ;
●－●, *Corynebacterium glutamicum* CG-2.

Protoplast fusion

The molecular weight and concentration of PEG as an material of cell fusion are different at every bacterium. Sovoda et al[15]. reported that 6,000 molecular weight was better than 4,000 in the protoplast fusion of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, and the 6,000 molecular weight of PEG was effective on *B. subtilis, B. flavum* and *Streptomyces coelicolor* in the protoplast fusion of bacterium since the attempt of Foddor and Alfoldi[6] on *B. megaterium*. The study of this experiment showed 1.8×10^{-5} frequency of fusion in the fusion of *B. flavum* BF-5 and *B. subtilis* BS-7 at the 30% concentration for the case of 4.000 molecular weight through the examination of molecular weight and

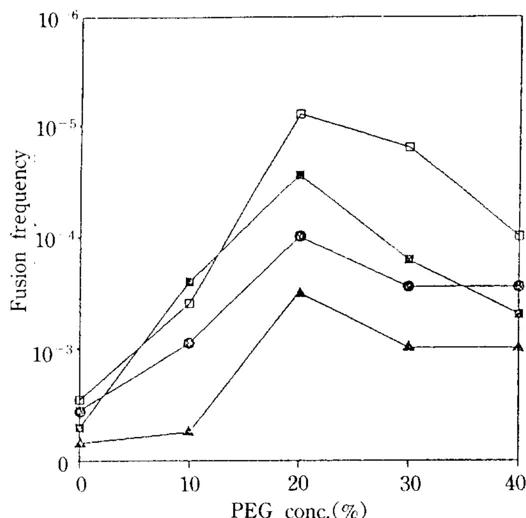

Fig. 3. Effect of PEG concentration no protoplast fusion
Protoplasts(2×10^8 of each auxotroph were treated with PEG at different concentration dissolved in 50 mM $CaCl_2$ at pH 6.5(30℃, 10min).
□－□ : *B. flavum* BF-5 and *B. subtilis* BS-7 treated with PEG 6,000
■－■ : *C. gulutamicum* CG-2 and *B. subtilis* BD 224 treated with PEG 6,000
●－● : *B. flavum* BF-5 and *B. subtilis* BS-7 treated with PEG 4,000
▲－▲ : *C. glutamicum* CG-2 and *B. subtilis* BD 224 treated with PEG 4,000

concentration of PEG in the intergeneric cell fusion, and 7.6×10^{-6} frequency of fusion for the case of *C. glutamicum* CG-2 and *B. subtilis* BD224. On the contrary, the maximum fusion frequency was appeared at 30% concentration and increased about 10 times in 6,000 molecular weight.

Characteristics of L-lysine producing fusants (Supplement of amino acid in nutrient requirement)

The biochemical and genetic methods are used for the selection of fused cell from the parental cells involving protoplast fusion. In this study, as the different nutrient requirement characters was used as a genetic selection marker, the supplement of mutual nutrient requirement was examined as an identification method of fused cell from intergeneric protoplast fusion. This study resulted the supplement of nutrient requirement of parental cells as shown on Table 2.

Table 2. Complementation test for confirmation of fusants

Strains	Hse	Leu	Met	AEC^r	Km^r
B. flavum BF-5	−		−	+	+
B. sutilis BS-7	−	+	−	−	+
FBB-41[a]	−	+	+	+	+
FBB-45	−	+	+	+	±

Strains	Thr	Trp	AEC^r
C. glutamicum CG-2	−	+	+
B. subtilis BD 224	−	−	−
FCB-15[b]	−	+	+
FCB-37	−	+	+

a) FBB : Fusants from *B. flavum* BF-5 and *B. subtilis* BS-7.

b) FCB : Fusants from *C. glutamicum* CG-2 and *B. subtilis* BD 224.

Growth rate of fusants

Comparing the growth degree from each intergeneric protoplast fusion to parental cells, the growth rate of fused cell, was rather increased than parental cell, a nutrient requirement strain.

Examination of AEC resistance

C. glutamicum CG-2 and *B. flavum* BF-5 strain have the resistance to AEC. As the AEC resistance test performed in the fusant with the following result on Table 3. Generally, AEC resistance of fusants became rather weak than the one of parental cell except FBB-41 fusant. It is one of the general characters of fusant and coincides with the result that the essential growth factor like an amino acid nutrient requirement is intent to be completely supplemented but the supplement is lowered in the secondary character like a analog resistance.

Table 3. AEC resistance of parents and fusants determined by cell growth

Strains	Conc. of AEC(mg/mℓ)			
	0	0.5	1.0	3.0
B. flavum BF-5	+++	+++	+++	+++
C. gulutamicum CG-2	+++	+++	+++	++
B. subtilis BS-7	+++	±	−	−
B. sutilis BD 224	+++	±	−	−
FBB-41	+++	+++	+++	++
FBB-45	+++	++	±	−
FCB-15	+++	+++	+	
FCB-37	+++	+++	+++	+

a) +++ denotes the level of growth rate comparable to the growth on the complete media without AEC.

+++ : Excellent ++ : Good + : Fair
+ : Poor − : No

Genetic stability of fusant

Genetic stability was examined on the L-lysine producing fusants appeared in the minimal cul-

ture media helping mutually between correlative nutrient requirement of parent strain and AEC and kanamycin resistance. The result of segregation rate test returning to parental strain is as shown on Table 4 when the fusant was transplanted in the complete culture media and the minimal culture media with tooth-pick method after cultivating it 4 times one week apart and breeding it in complete culture media and picking each 500 colony. FBB-41 strain was very stable fusant but FBB-45 was unstable one showing 24.4% segregation rate. It shows the difference from the stability of fusant from intergeneric protoplast fusion reported by Steward et al[14]. and Sovoda et al[15].

Table 4. Genetic stability of fusants

Strains	Colonies on CM	Colonies on AMM[a]	Segregation (%)
FBB-41[b]	500	400	0
FBB-45	500	378	24.4
FCB-15	500	495	1.0
FCB-37	500	498	0.4

The genetic stability of fusants was determined after keeping for 4 weeks at 4℃

a) AMM : MM containing AEC(1㎎/㎖) and kanamycin(50μg/㎖)

b) FBB and FCB are the same as showed in Table 2.

Aspartokinase activity of L-lysine producing fusants

As the result of key enzyme, aspartokinase activity, compairson test between parental strains and fusants in the process of lysine biosynthesis, the L-lysine producing fusant, FBB-41 increased 114.7% in enzyme activity compared with *B. flavum* BF-5 strain(Table 5). It coincides with the result of 114.7% increase of aspartokinase activity in the comparison between *B. flavum* BF-5 and *B. subtilis* BS-7 has genetic stability and more powerful aspartokinase activity related to L-lysine production than parental strains, it shows the possibility of lysine production from soluble starch as a source of carbon rather than glucose.

Table 5. Aspartokinase activity of parents and fusants

Strains	Specific activity (nmol/min/㎎ protein)	Relative activity (%)
B. flavum DF-5	17.8	100
B. subtilis BS-7	2.8	15.7
FBB-41[a]	20.4	114.7
FBB-45	18.2	102.2
C. glutamicum CG-2	17.3	97.2
B. subtilis BD 224	2.7	15.2
FCB-15	18.9	106.2
FCB-37	17.5	98.3

a) FBB and FCB are the same as showed in Table 2.

요 약

L-lysine 생산균주인 Coryneform bacteria와 전분분해능을 가지는 *Bacillus subtilis* 균주간의 원형질체 융합을 시도하여 전분에서의 *L-lysine* 생산 가능성을 실험하였다.

원형질체 생성에 있어 *Bacillus subtilis*의 경우 원형질체 형성은 lysozyme 처리만으로 가능하였으나 Coryneform bacteria는 대수증식기에 0.3 unit/㎖ 농도의 penicillin G를 처리한 후 lysozyme 처리하여 원형질체를 얻었다. 이들 이속간의 세포융합 빈도는 PEG 분자량 6,000의 30%농도에서 10^{-4}에서 10^{-5} 빈도를 보였으며, 융합체는 각각의 선택배지상에 나타나는 colony를 취하여 융합체로서의 성질로서 균의 생육도, 유전안정성, 영양요구성의 상호 보완성등을 실험하였다. 융합체 FBB-41의 경우, 양친 균주의 영양요구성이 상호보완되었으며 AEC내성과 kanamycine내성을 동시에 보유하였으나 AEC내성의 경우, 양친균주보다는 다소 약하였다. 또한, 융합체는 양친 균주보다 높은 aspartokinase 활성을 보였다.

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