Practice of Industrial Strain Improvement

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ABSTRACT—Industrial strain improvement is concerned with developing or modifying microorganisms used in production of commercially important fermentation products. The aim is to reduce the production cost by improving productivity of a strain and manipulating specific characteristics such as the ability to utilize cheaper raw materials or resist bacteriophages. The traditional empirical approach to strain improvement is mutation combined with selection and breeding techniques. It is still used by us to improve the productivity of organisms in amino acids, organic acids and enzymes production. The breeding of high L-lysine-producing strain Au112 is one of the outstanding examples of this approach. It is a homoserine auxotroph with AEC, TA double metabolic analogue resistant markers. The yield reaches 100 g/l. Besides, the citric acid-producing organism *Aspergillus niger*, Co827, its productivity reaches the advanced level in the world, is also the result of a series mutations expecially with ^{60}Co γ-radiation. The thermostable α-amylase producing strain A 4041 is the third example. By combining physical and chemical mutations, the strain A 4041 becomes an asporogenous, catabolite derepressed mutant with rifamycin resistant and methionine, arginine auxotroph markers. The α-amylase activity reaches 200 units/ml. The fourth successful example of mutation in strain improvement is the glucoamylase-producing strain *Aspergillus niger* SP56, its enzyme activity is 20,000 units/ml, 4 times of that of the parental strain UV-11. Recently, recombinant DNA approach provides a worthwhile alternative strategy to industrial strain improvement. This technique had been used by us to increase the thermostable α-amylase production and on some genetic researches.

Introduction

The importance of microbial strain is selfevident for fermentation process. The core of traditional industrial strain improvement is isolating and screening mutants with desired characteristics (including productivity increased, ability to use cheaper raw materials, filtration property improved or bacteriophages resisted, etc.). The selection of spontaneous mutation is one way to get mutants with improved properties. And more important, the mutagenesis combined with fermentation screening process is an essential mean of improving most industrial fermentation process. The advantages of mutation combined with fermentation screening process are simple, most direct and least expensive. Besides, it requires little knowledges on genetics, physiology and the important pathway involved in the biosynthesis of the desired products. The aim of mutagenesis is to increase mutation rate with physical mutagen (e.d. ultraviolet, gamma and X-irradiation) and chemical mutagen (e.g. MNNG and DES). The technical manipulation is easy to deal N-methyl-N-nitroso-N′-nitroguanidine, diethyl sulphate with.

Recently, recombinant DNA approach provides a worthwhile alternative strategy to industrial strain improvement. Genetic engineering allows us to manipulate specific genes in a controlled way. It has powerful potential as a strain improvement technique.

In the past 25 years after our institute founded

in 1966, the traditional approach of industrial strain improvement had been used efficiently by us to select excellent mutants e.g. glucoamylase producing strains, citric acid producing strains, L-lysine producing strains, inosine producing strains and guanosine producing strains. All the excellent mutants had brought us a great deal of economic benifits already. The practical works of industrial strain improvement in our institute will be introduced in the following, as well as some preliminary works we had tried on genetic breeding.

I. Practice of Industrial Strain Improvement by Traditional Approaches

Spontaneous mutation and physico-chemical mutation are used most commonly among various means of mutation. In fact, excellent producing mutants with desired characteristics are often derived from wild strains by mutation in the practice of industrial production. Mutation with physical mutagen and chemical mutagen are easy to deal with, the results are effective and direct, no expensive equipments and devices are required. The followings are some successful examples in our institute.

1. The Mutation and Selection of Glucoamylase Producing strain *Aspergillus niger* SP56

In 1977, we got the mutant UV-11 of *Aspergillus niger* from Institute Microbiology, Academic Sinica, Beijing. After a series of mutations, some excellent strains were derevied from UV-11, e.g. UV-111, γ-471, P-10 and SP-56. The enzyme activity was increased from 5,000 u/ml of the parental strain to 20,000 u/ml of P-10, reached the most advanced level at homeland (Figure 1).

2. The Mutation and Selection of Citric Acid Producing Strains *Aspergillus niger* 3008 and Co 827

In order to raise fermentation level of citric acid in our country, we specialized the research on selection of citric acid producing strains. More than 700 soil samples were collected from different parts of the country. From them, about 7000 strains which had the abilities to resist acids were

Fig. 1. Mutation and Selection of Glucoamylase Producers

Year	Strain	Enzyme activity
1977	*Aspergilus niger* UV-11	5000 u/ml
	MNNG	
1980	UV-111	10000 u/ml
	^{60}Co, MMNG	
1983	γ-471	10000 u/ml
	MMNG	
1986	P-10	15000 u/ml
	MNNG	
1991	SP-56	20000 u/ml

screened. After assaying citric acid yield, 40 around citric acid overproducing strains were obtained. Among which the highest citric acid yield were 3-6%. By mutation of ^{60}Co, γ-ray of 80,000-100,000 Rontgen (100 R/S) and empirical screening, 6 mutants were selected. Among which, mutant D353 of *Aspergillus niger* was the best, it's citric acid yield was over 9%. On the scale of 5,000 l fermenter, 16% sweet potato powder as medium, temperature of 33℃, after 5 days, citric acid yield reached 9%, conversion rate 90%.

In 1979, based on the experience we learned from the selection research of citric acid producing strain with sweet potato powder as raw material, we discovered that the citric acid production by fermentation was closely related to microorganism's morpha and size. By the experience, again with ^{60}Co, γ-ray, we got the excellent mutant *A. niger* 3008. In the scale of 5,000 l fermenter, sweet potato powder as the sole medium, citric acid yield reached 18-20%, conversion rate 90%, productivity 30 kg/m^3/d. Because of its outstanding characteristics the mutant 3008 was widely adopted in China's fermentation factories to produce citric acid.

In 1982, new strain *A. niger* Co827 derived from

Fig. 2. Mutation and Selection of Citric Acid Producers

Year	Strain	Yield (%)	Convertion rate(%)
1974	Wild *Aspergillus niger* (Isolated from Soil)	5-6%	
	^{60}Co, γ-ray		
1974	Mutant D353	9%	90%
	^{60}Co, γ-ray		
1979	Mutant 3008	18%	90%
	^{60}Co, γ-ray		
1982	Mutant Co827	15%	95%

Fig. 3. Mutation and Selection of L-Lysine Producers

Year	Strain	Shake flask Yield(%)	Fermenter Yield(%)
1978	Wild Strain 2305		
1978	H-2 (HS$^-$)	3-3.5	
1980	A111 (HS$^-$, AEC^r, Urea$^-$)	5-5.5	4.4-4.8(0.5 m^3)
1983	Au-111-2 (HS$^-$, AEC^r, Urea$^-$)	6-6.5	5-5.5(12 m^3)
1986	Au112 (HS$^-$, AEC^r, TA^r, Urea$^-$)	8-10	12-15(16 m^3) 9-10.6(30 m^3)

mutant 3008 by mutation with diethylsulfate. Its citric acid yield reached 16% with starch as raw material, conversion rate 95%, fermentation time 115.6 h. Using sweet potato powder as raw material was available too, then the citric acid yield was more than 15%, conversion rate 95% over, fermentation time 60 h. We have the honour to say that citric acid production in China had been increased greatly because of new strains 3008 and Co827. China had been changed from importer of citric acid into exporter of citric acid afterwards (Figure 2).

3. The Mutation and Selection of L-Lysine Producing Strain AU-112 of *Corynebacterium glutamicum*

In 1978, by mutation of *Corynebacterium glutamicum* 2305, a wild strain, some auxotrophic mutants were derived. Among them, a homoserine auxotrophic mutant H-2 (HS$^-$) was screened and selected, its L-lysine yield reached 3-3.5%, conversion rate was 25-30%. In 1979, derived from mutant H-2, a mutant A111 (HS$^-$, AEC^r, Urea$^-$) was achieved. Its L-lysine yield were 5-5.5% (shake flask) and 4.5-4.7% (500 L fermenter). The total extraction yield was 63.1%, on the scale of 500 L fermenter, reachd the most advanced level in China at that time.

In 1983, derived from AU-111, a new mutant of AU-111-2 (HS$^-$, AEC^r, Urea$^-$) was selected. In shake flask, its L-lysine yield was 6-6.5%, conversion rate 36-40%. On th scale of 12 m^3 fermenter, average fermentation time of 5 batches was 53.8 h, yield 5-5.5%, conversion rate 30-34%, total extraction yield 70.26%, reached the most advanced level at homeland again.

In 1986, derived from AU-111-2 by mutation, an excellent strain of mutant AU-112 (HS$^-$, AEC^r, TA^r, Urea$^-$) was obtained. With glucose or starch hydrolyzate as raw material in shake flask, L-lysine yield was 8-9%, conversion rate 40-50%. On the scale o 15-20 l autocontroled fermenter, with fed batch of sucrose and ammonium liquor, the yield was 12-15%, conversion rate 34-42%, total extraction yield 86.9%. On the scale of 12 L fermenter, fermentation time 50-60 h. L-lysine yield 6-7.3%, conversion rate 30-36%. As a Sino-French co-opertive research, the L-lysine production of AU-112 was carried out in France, the L-lysine yield was 9-10.6% on the scale of 30 m^3 fermenter (Figure 3).

4. The Mutation and Selection of Alkaline Proteinase Producing Strain JK38 Which Resists Phages—An Example of Spontaneous S-(2-Aminoethyl)-L-Cysteine. Thiazolealanine Mutation

Bacillus licheniformis 2709 JC-10 was an important producer of alkaline proteinase in China's fe-

Fig. 4. Selection of Phage Resistant Strain JK38

Strain	Enzyme activity
Bacillus licheniformis 2709 (Easy Contaminated by Phage)	4500 u/ml
Phage resistant Strain JK38 (Phage resistant0	4500 u/ml

rmentation factories before 1974. It was comtaminated by phages from time to time resulting serious losses in production. Shanghai Newtype Fermentation factory, Fudan University and our Institute carried on cooperatively a research to overcome the problem. By the mean of spontaneous mutation, a phage resistant mutant JK38 was achieved from sensitive strain 2709-JC10, it could resist phage P747 which was isolated from the comtaminated broth. On the scale of 5 m^3, 10 m^3 and 20 m^3 fermenters, the enzyme activity reached 4,500 u/ml, the same as that of the original strain (Figure 4).

5. The Mutation and Selection of 5′-IMP Producing Strain 265

5′-IMP has a palatable taste, which was produced by enzymatic synthesis or fermentation in Japan the early year of 1960's. In the middle of 60's, our institute began its research of 5′-IMP fermentation production with *Corynebacterium glutamicum* 2305. Based on the theory of biosynthetic pathway of purine nucleotide in this glutamic acid producing strain, it had to get a mutant with genetic marker of adenine deficiency. With mutation by UV and DES, a mutant 265 (ade$^-$, Met$^-$, His$^-$, Nicotinic acid$^-$) was derived. On the scale of 5,000 L fermenter, the highest yield of 5′-IMP reached 4.63 g/l, the average yield was 3.87 g/l. This is a typical example of intermediate metabolite production by auxotrophic mutant (Figure 3).

6. Mutation and Selection of Thermostable α-amylase Producing Strain A4041

Thermostable α-amylase is an important enzyme, its production researches are widely specia-

Fig. 5. Mutation and Selection of 5′-IMP Producers

Strain	Yield (g/l)
Glutamic acid Producer 2305	
UV Mutant A_1 (Ade$^-$)	5′-IMP 1 g/l
UV Mutant B_2 (Ade$^-$, Met$^-$)	5′-IMP 2 g/l
DES Mutant C_3 (Ade$^-$, Met$^-$, His$^-$)	5′-IMP 3 g/l
DES Mutant 265 (Ade$^-$, Met$^-$, His$^-$, Nicotinic acid$^-$)	5′-IMP 4.6 g/l

Fig. 6. Mutation and Selection of Thermostable α-amylase Producers

Strain	Enzyme activity
Bacillus licheniformis B198	10-20 u/ml
NTG, DES, UV, ^{60}Co, γ-ray, NTG+UV	
Mutant A4041 (Rif^r, Met$^-$, Arg$^-$)	150-200 u/ml

lized in abroad and homeland. In 1980, start from *Bacillus licheniformis* B198, by mutation of NTG, DES, UV, ^{60}Co, γ-ray and NTG+UV, a mutant A4041 (Rif^r, Met$^-$, Arg$^-$) was obtained. Its enzyme activity was 100 fold of that of B198. In shake flask, enzyme activity reached 200 u/ml. On the scale of 800 l fermenter, fermentation time 96 h, enzyme activity reached 150 u/ml. Scaled up to 2,000 L fermenter, fermentation time 105 h, enzyme activity was 150 u/ml (Figure 6 and Table 1).

7. Mutation and Selection of Alkaline Proteinase Producing Strain: Application of Protoplast Mutation

In China, alkaline proteinase together with α-amylase and glucoamylase are the 3 most important enzymes. Their productions and demands in China's market are nearly the same. The *Bacillus licheniformis* 2709 has been used as a production

Table 1. The Mutagenesis Pedigree from Strain B.198 to Mutant A.4041

Strains	Mutagen(final concentration)	Survival rate(%)	α-amylase activity (BV×m³/ml)	Markers
B.198	NTG 200 γ/ml, S*	0.535	10-20	−, AP 196 u/ml
88-8	NTG 200 γ/ml, S NTG 40 γ/ml	0.156	40-50	Spo
76-14-18	++S rif 5 γ/ml	1.4	80-90	Spo
A.915-7	UV 20″, S	3.3	130	Spo , Rif^r
A.1372	NTG 4 γml, S	18	130	Spo , Rif^r
A.1788	NTG 100 γ/ml, S	0.15	150	Spo , Rif^r
A.2200-11	NTG 100 γ/ml, S NTG 100 γ/ml	4.8	250	Spo , Rif^r, Met , Ap1840 u/ml**
A.2661-9	+S UV 9″	0.45×10^{-2}	264	Spo , Rif^r, Met
A.3052-7	NTG 200 γ/ml, S	0.18	275	Spo , Rif^r, Met
A.3351-6-5	DES 1%, S	2.7×10^{-2}	320-370	Spo , Rif^r, Met
A.3617-2-6	Co^{60} γ-ray 10^5 rad		350-380	Spo , Rif^r, Met
A.4041	Co^{60} γ-ray 6×10^4 rad	0.1	450	Spo , Rif^r, Met , Arg , AP1616 u/ml
A.4808	S		300	Spo , Rif^r, Met , Arg , Cyc^r
A.4808-3	—	—	330-360	Spo , Rif^r, Met , Arg , Cyc^r

*S : Selection, **AP : Alkali protease activity.

strain in our country, the enzyme activity reached 7,000-8,000 u/ml in submerged fermentation. In order to improve its fermentation production, we tried the protoplast mutation to select an overproducing strain. Treating protoplast by NTG, an overproducing mutant A-57 was derived. After 40 h fermentation in shake flask, enzyme activity reached 13,000 u/ml around. In the scale of 2,000 L fermenter, average enzyme productivity was 13,000 u/ml, the highest was 15,000 u/ml. On 25,000 L fermenter, average enzyme activity was 12,000 u/ml, the highest was 13,000 u/ml, the enzyme activity increased 70% of that of original strain 2709. Moreover, A-57 was stable in enzyme production, it was an excellent producer of thermostable α-amylase (Figure 7).

Fig. 7. Mutation and Selection of Alkaline Protease Producer A-57

Strain	Enzyme activity
Bacillus licheniformis 2709	7000-8000 u/ml
Protoplast treated with NTG mutation	
Mutant A-57	13000 u/ml

II. Protoplast Fusion and Preliminary Researches of Recombinant DNA Technique

The disadvantages of traditional mutation and selection are wasting time, because of it's great amount of work have to be done and its strain screening in random way. In recent years, new techniques in biotechnology, e.g. cell fusion and genetic engineering, applied in microorganism breeding, result a prosperous future for improving production level and new fermentation products developing. We are sure to say that new biotechnology will be a great impact against the old fermentation industry and provide a chance to develop for the latter.

1. Improving Thermostable α-amylase Producing Strain with Protoplast Fusion Technique

Protoplast fusion is a simple technique relatively in genetic engineering. We tried it to improve thermostable α-amylase producing strain A4041.

Fig. 8. Enzyme Production by the Protoplast Fusant

Strain	PH in fermentation broth	Enzyme activity (u/ml)	Enzyme activity increased
Parenal strain A4041	6.5	40	—
Parenal strain PF1093	8.0	30	—
the Fusant	6.0-6.5	60-70	50-70%

With the protoplast fusion technique, a fusant of double resistant prototroph was achieved from parental strains mutant A4041-E of *Bacillus licheniformis* (Met⁻, Arg⁻, Em^r, Cyc^s) and *Bacillus licheniformis* PF1093 (Met$^+$, Arg$^+$, Em^s, Cyc^r). There was no morphological variation between the fusant and its parental strains. But morphological changes clearly observed by naked eyes after plate cultured in complete medium and minimal medium with EM (2 μg/ml) Cyc (60 μg/ml). The parental strain A4041-E was the colour of white grey with dark red and grew slowly. PF 1093 was the colour of white grey and grew quickly, but the fusant was the colour of orange red and grew more quickly. The advantage of the fusant was its enzyme activity increased 50-75% Erythromycin, Cycloserine to that of its parents. Moreover, the fusant could be reserved much longer than its parental strains. After half a year reservation, the enzyme activity of the fusant still reached 60 μg/ml with regeneration. This was the successful example of changing genetic characteristics with protoplast fusion technique (Figure 8).

2. Researches of *Corynebacterium gutamicum* with Recombinant DNA Technique

(1) Detection of Plasmid DNA in Industrial Strains

Plasmid is an important gene vector in recombinant DNA technique, it can be selfreplicated outside chromosome. Since there are more than one copy number (sometimes over 100) in a cell, we can use it to make gene amplified to meet the requirement of improving fermentation products and their yields. In order to look for an efficient system of plasmid vector, we cooperated with Research Institute of Genetics in Fudan University, to carry out the work in detection of plasmid DNA in industrial strains. As a result, we founded a new method for rapid determination of plasmid DNA in microorganisms. It was based on Eckhardt method and improved the latter. Application of the new method, we found 9 strains which carried plasmid DNA in all the 112 strains we collected (Table 2).

As the Table 2 indicated, plasmid existed in *Corynebacterium glutamicum* and *Aerobacter aerogenes*. This discovery was reported first time in China, it had a great significance for discovering plasmid existed in *C. glutamicum* especially. At that time, only plasmid of *Bacillus* sp. was used as gene vector among Gram-positive bacteria. As a glutamic acid producer and other amino acids or nucleotide producers, th nonpathogenic *Coryne-*

Table 2. The Strains Carried Plasmid

No.	Original No.	Strain	Products
1	750608	*Corynebacterium glutamicum*	Glutamic acid
2	1014	*Corynebacterium glutamicum*	Glutamic acid
3	20	*Bacillus* sp.	Glutamic acid
4	94	*C. glutamicum*	Glutamic acid
5	B9	*B. megaterium*	—
6	B14	*Escherichia coli*	Decarboxylase for Glutamic acid determination
7	B83	*Escherichia coli*	—
8	B96	*Aerobacter aerogenes*	Isoamylase
9	B167	*B. natto*	α-amylase

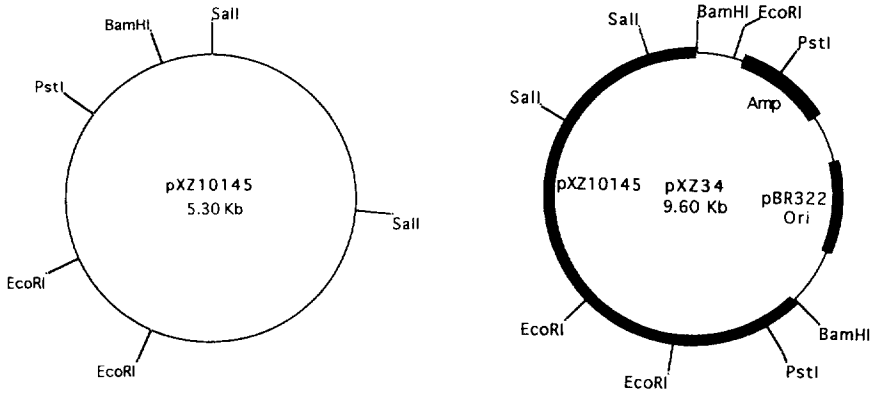

Fig. 9. The Restriction Map of the Plasmid pXZ10145 and Plasmid pXZ34.

bacterium glutamicum has great economic value. Research on plasmid of *C. glutamicum* provides an important way to improving production properties of this kind of bacteria.

(2) Research on Plasmid pXZ10145 of *Corynebacterium glutamicum*

As the Plasmid of *C. glutamicum* was important in theory research and practical application, the characteristic of plasmid had been specialized since the discovery of plasmid pXZ10145 in *C. glutamicum* 10145. First, learnt that the plasmid carried chloramphenicol resistant marker. Through electronic microscope determination, the size of plasmid pXZ10145 was 5.3 Kb. Digested with restriction enzymes *Bam*HI, *Pst*I, *Eco*RI, *Sal*I, and double digested with *Bam*HI+*Sal*I, *Bam*HI+*Pst*I, *Pst*I+*Sal*I, *Eco*RI+*Pst*I, *Eco*RI+*Bam*HI, the restriction enzyme map of pXZ10145 was achieved. The map verified that *Bam*HI and *Pst*I were the single enzyme digestion points. See the left of Figure 9. Linked this plasmid and pBR322 with digestion point *Bam*HI, the chimeric plasmid pXZ34 was obtained. See the right of Figure 9. The latter could be replicated in *E. coli*. The aminobenzylpenicillin and chloramphenicol resistant markers could be expressed in *E. coli*. But the chloramphenicol resistant capability was decreased a lot, the tolerance only 2 γ/ml.

3. Application of Gene Clone and its Expression

(1) The Cloning of a β-cyclodextrin glucanotransferase gene

(β-CGTase gene) from an alkalophilic Bacillus N-227 and its expression in *E. coli*.

The cloned gene was located on a 4.0 Kb *Hin*dIII-*Sal*I fragment and was subcloned into a shuttle vector pN3 which was constructed from pUB 110 and pBR322 carrying the promoter and signal peptide sequence of a thermostable α-amylase gene. We attempt to get the high expression of the β-CGTase gene with the strong promoter of the α-amylase gene.

(2) Cloning and expression of the Enterobacter aerogenes W8401 purine nucleoside phosphoryase gene in *E. coli*

The PNPase is the key enzyme in enzymatic synthesis of virazol and other virustatic nucleosides. The PNPase gene was located on a 1.2 Kb *Sau*3A-*Pvu*II fragment. The gene was sequenced by Sanger Dideoxy mediated Chain termination method. We found an open heading frame of approximately 600 base pairs which encoded a poly peptide of 200 amino acids. An inducible expression Vector pJLA602 allowed the high level expression of the PNPase gene.

(3) The cloning of the 3-deoxy-D-arabino-hepturosonate-7-phosphate (DAHP) synthese gene and the prephenate dehydratase gene in *corynebacterium glutamicum* and their application to the breeding of a L-phenylalanine producer.

Prephenate dehydratase and DAHP synthase are two key enzymes that regulate the L-phenyla-

lanine production. We try to clone these two genes with a vector pBZ51 and select the positive transformants by auxotroph complementation. We have already constructed a series of expression vector systems that can be used in corynebacterium on high level.

This work is under way.

Conclusion

Indicated by examples mentioned above, traditional mutation breeding is still the major approach in industrial strain improving. By progress in mutation screening and selection, awareness of mutants desired in the procedure, the traditional approach of mutation and selection will be optimized. The result will promote fermentation industry to develop greatly.

As a supplement and innovation, recombinant DNA will speed up the progress in microorganism breeding without a doubt, resulting the present fermentation products and yields increased, production cost reduced. The high technique of genetic engineering is creating some new organisms which are not existing in the nature, mankind will utilize these new organisms to produce necessary medicines and varieties of product which could not be produced by present techniques to meet the demand of market. Increasing new strains by genetic engineering, constructing more and stable vector systems, looking for more efficient transformation methods and higher expressions, will be the specialized subjects in genetic engineering.