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Improvement of *Aspergillus niger* 55, a Raw Corn Meal Saccharifying Enzyme Hyperproducer, through Mutation and Selective Screening Techniques

Oh, Sung-Hoon and Pyong-Su O*
Fermentation Technology Laboratory, R & D Center, Pacific Chemical Co. Ltd., Ansan 425-120, Korea

옥수수 生 전분 당화 효소 高 생산성 변이주 개발

오성훈 · 오평수*
태평양화학(주) 기술연구소 발효공학연구실

Abstract — Mutation experiments were performed to select the mutant of *Aspergillus niger* 55, which had lost almost all the ability to produce transglucosidases but retained that of high productivity of raw corn meal saccharifying enzyme, by means of successive induction with N-methyl-N'-nitro-N-nitrosoguanidine(MNNG), ultraviolet(UV) light, and γ-rays. Also, we used the mutant enrichment techniques, such as liquid culture-filtration procedure and differential heat sensitivity of conidia, in order to increase the possibility of obtaining a mutant. The glucoamylase productivity of mutant PFST-38 was 11 times higher than that of the parent strain. The mutant PFST-38 was morphologically identical to the parent strain, except for the size of conidia, the tendency to form conidia and the length of conidiophore. *Asp. niger* mutant PFST-38 appeared to be useful for the submerged production of the raw corn meal saccharifying enzyme.

We isolated a fungus, *Aspergillus niger* 55, which was a superior raw corn meal saccharifying enzyme producer in previous studies (1). However, the enzyme productivity of this fungus was not adequate for industrial application. The commercial importance of the glucoamylase is well recognized. Of the several microbial strains known to produce this enzyme, species of *Aspergillus* such as *Asp. awamori*, *Asp. niger* and *Asp. faetidus* have been used for obtaining the enzyme on an industrial scale. For commercial production of glucoamylase, attempts were made to obtain potent mutants from a powerful glucoamylase-producing strain, *Asp. niger* 55. Glucoamylase synthesis is known to be accompanied by the formation of transglucosidase, which adversely affects the efficiency of saccharification. Transglucosidase promotes the formation, particularly from maltose and glucose, of unfermentable carbohydrates. When transglucosidase is present as a contaminant in glucoamylase employed to hydrolyze starch to dextrose, lower yields of dextrose are obtained. Large amounts of glucoamylase can be produced by *Asp. awamori* without objectionable quantities of transglucosidase (2-6). This paper demonstrates induction of a mutant of *Asp. awamori* by chemical and physical means that produces a large amount of glucoamylase without formation of transglucosidase.

In the studies reported here, we describe the breeding of a mutant from *Asp. niger* 55, which has lost almost all the ability to produce transglucosidases but retained that of high productivity of raw

Key words: *Asp. niger* mutant PFST-38, raw corn meal saccharifying enzyme, glucoamylase, transglucosidase
*Corresponding author

corn meal saccharifying enzyme, by means of successive induction with MNNG, UV and γ-rays. And also, we describe the mutant enrichment procedure used, such as the minimal medium liquid culture-filtration procedure in order to increase the possibility of obtaining a mutant. The productivity of glucoamylase was found to be several times that of the parental strain. Furthermore, we also found that some relationships existed between the high productivity of the enzymes and certain morphological characteristics of the mutants, such as the tendency to form conidia, the size of conidia and the length of the conidiophore.

Materials and Methods

Media and growth conditions

Minimal medium for agar plates contained: 1.5% (w/v) agar; 3% (w/v) glucose; 2.0 mg asparagine/m*l*; 1.5 mg KH_2PO_4/m*l*; 0.5 mg $MgSO_4$/m*l*; and 0.5 μg thiamine/m*l*. Minimal medium for liquid cultures was identical except for the omission of agar. Enriched complete medium for agar plates contained 2.5% (w/v) agar; 2.0% (w/v) raw corn meal; 0.14% (w/v) $(NH_4)_2 SO_4$; 0.03% (w/v) urea; 0.2% (w/v) KH_2PO_4; 0.03% (w/v) $CaCl_2$; 0.03% (w/v) $MgSO_4 \cdot 7H_2O$; 0.5% (w/v) polypeptone; 0.1% (w/v) yeast extract; and 0.3 m*l* Triton X-100. In complete medium for liquid cultures, the agar and Triton X-100 were replaced by 2.0 g/*l* of Tween 80. Raw corn meal was sterilized with the method described in a previous paper (7). Liquid cultures were grown for 20 to 48 hrs at 30℃ with aeration.

Mutagenesis

MNNG treatment: *Asp. niger* 55 was used as the parental strain. This strain was cultured at 25℃ for 10 days on Czapek-Dox agar medium. Conidia were heat-shocked at 48℃ for 15 min and then sedimented to give pellets containing 10^7 conidia. N-methyl-N′-nitro-N-nitroso-guanidine was dissolved in 0.2 M citrate buffer, pH 5.0, and its concentration adjusted to 4.0 mg/m*l*. MNNG solution was added to the conidia suspension (1.0 mg MNNG solution per 1 m*l* conidia suspension) and the mixture was allowed to stand for 30 min without shaking during the treatment period (killing rate of conidia was above 99.9%). Then the conidia were washed three times by centrifuging (3000 rev./min) and resuspended in sterile water. After the final wash, 1 m*l* of the conidia suspension was transferred into 50 m*l* of minimal medium (Fig. 1).

Ultraviolet irradiation: 10 m*l* of the conidia suspension (about 10^6 conidia/m*l*) were transferred into a Petri dish and irradiated with an ultraviolet lamp (15 W) at a distance of 30 cm. After irradiating for 15 min (survival rate less than 1%), 1 m*l* of the conidia suspension was transferred into minimal medium.

γ-Ray irradiation: The conidia formed were collected and suspended in 0.1% sterilized peptone solution in a 10 m*l*-cap tube with a density of about 10^5 conidia/m*l* and irradiated with γ-rays from a Cobalt-60 source for 50 minutes. The radiation dose at the distance used was 1,000 γ/min, totaling, therefore, 50,000 γ. Survival was somewhat less than 0.15 percent.

Mutant enrichment: A minimal medium liquid culture-filtration procedure facilitated mutant cell enrichment. Only wild type cells grow in the minimal medium while ungerminated, presumably mutant, conidia can be separated from these by filtration. Although liquid culture-filtration selection has been successful in enriching mutants, repeated cycles were necessary to eliminate wild type cells. Mutagenic treatment apparently delayed cell division in a treated population so that differences between wild type cells and mutants developed only after several selective cycles. Even when conidia failed to germinate in a minimal medium liquid culture, they would often appear on minimal medium agar plates. Therefore, by using the differential heat sensitivity between ungerminated mutant conidia and germinated wild type conidia, it is possible to increase the proportion of mutant. Germinated conidia and mycelia die when left at temperatures higher than about 45℃ for 24 hrs but ungerminated conidia survive this treatment and subsequently germinate if transferred to complete medium.

Screening of mutants

The mutagen treated conidia were plated onto

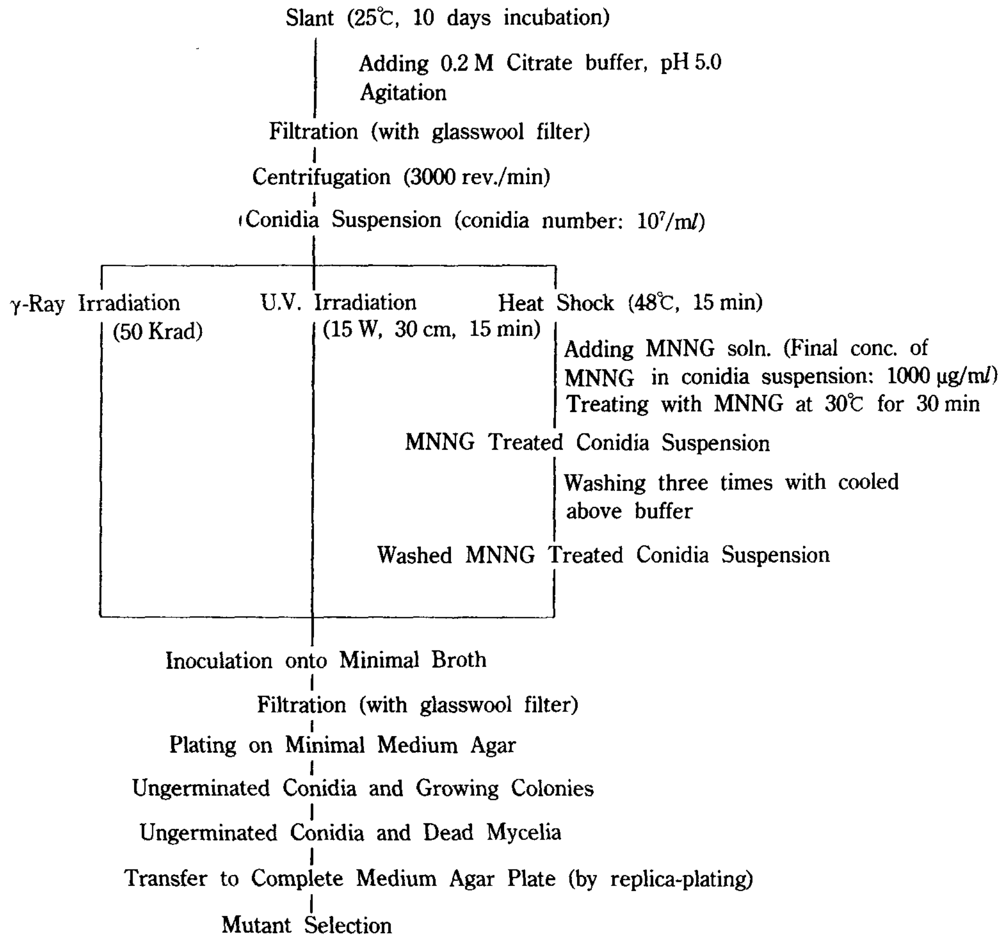

Fig. 1. The methods of mutagen treatment and mutant enrichment.

the complete medium, and incubated at 30℃ for 4 days. The agar blocks displaying clear zones around them were used for further investigation. The initial screening was based on the ratio (*R*) of the diameter of the clearance to the diameter of the agar-block. The colonies with significantly higher values of *R* as compared with the parent were selected, and were assayed for the activity of enzymes. Mutants with significantly higher glucoamylase activities were selected and further subjected to the combined action of MNNG, UV and γ-ray treatments. The stable mutants were again further tested for their ability to produce glucoamylase.

Growth in liquid medium and preparation of crude enzyme

Conidia from purified strains were inoculated into 5 m*l* of the complete medium in a test tube followed by incubation at 35℃ for 24 hrs, then the culture was transferred to 100 m*l* of the complete medium in a 500-m*l* Sakaguchi flask and the incubation was continued on a reciprocal shaker. After cultivation, the cells were removed by filtration through filter paper (Whatman No. 2), and the filtrate was used as the crude enzyme preparation.

Assay of enzyme activity

RDA: Raw starch digesting-ability (RDA) was determined by measuring starch saccharifying activity according to the method of Oh *et al.* (7). Raw starch digesting activity was assayed using a reac-

tion mixture consisting of 20 mg of corn meal, 0.2 *ml* of 0.1 M acetate buffer solution (pH 4.5) and 1.6 *ml* of distilled water.

Glucoamylase activity: Glucoamylase activity was measured in a reaction mixture that contained 0.5 *ml* of 2% soluble starch in 0.2 M sodium acetate buffer (pH 4.8) and 0.5 *ml* of enzyme solution. After aerobic incubation at 60℃ for 30 min, the reaction was stopped by cooling the mixture on ice, and then the mixture was boiled in a steam bath for 10 min. The released glucose was quantified by using the hexokinase-glucose-6-phosphate dehydrogenase method (8). One unit of glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per min under the assay conditions used.

Transglucosidase activity: A solution of maltose was prepared by dissolving 100.0 g C. P. maltose in distilled water and diluting to 500 *ml*. A 50.0 *ml* portion of this 20 weight percent maltose solution was then placed in a 100 *ml* flask and diluted to 100 *ml* with distilled water. To the flask was added 5 *ml* of 1.0 M acetate buffer, pH 4.5. After mixing, an amount of enzyme preparation containing 5.0 units of glucoamylase activity was added. The flask was placed in a 55℃ water bath for 48 hrs. At the end of this incubation period, the optical rotation of the sugar solution was measured. The higher the specific rotation measured at 25℃, $[\alpha_D^{25}]$, the higher the transglucosidase activity or content of the enzyme preparation being tested.

Cultivation in jar fermentor

Mutant PFST-38 was pregrown in a medium A containing per liter of distilled water: 25 g soluble starch, 30 g peptone, 5 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_2O$. The glucoamylase production medium B contained per liter of distilled water: 10 g corn meal, 20 g rice bran, 30 g wheat bran, 1 g HCl (36%), 1 g $NaNO_3$. After inoculation of conidia, shaking cultivation was carried out in a 1 *l* Sakaguchi flask containing 100 *ml* of the medium A on a reciprocating shaker with agitation at 35℃ for 24 hrs. Jar cultivation was started by transferring 300 *ml* of a conidial suspension into 15 *l* of the medium supplemented with 0.05% silicon as an antifoam in a 30 *l* jar fermentor (Marubishi Co.) and continued under forced aeration at 1 vvm and stirring at 400 rpm. Cultivation was carried out at 30℃ for 5 days.

Analytical methods

Mycelia and broth of the liquid culture (10 *ml*) were separated by centrifugation and filtration with paper (Whatman No. 2). Mycelial dry weight was determined after the mycelia had been washed three times with distilled water and dried. Total sugar was determined using the Phenol-Sulfuric acid method described by Dubois (9).

Results and Discussion

Selection of mutants

Four typical mutants representing different stages of induction were obtained by successive induction processes. The sequence of mutation is shown is Fig. 2. At first, only 45 isolates with high values of *R* as compared with the parent were selected. These isolates were further tested for their ability to produce glucoamylase and transglucosidase. One clone (P-72) exhibiting the lowest transglucosidase

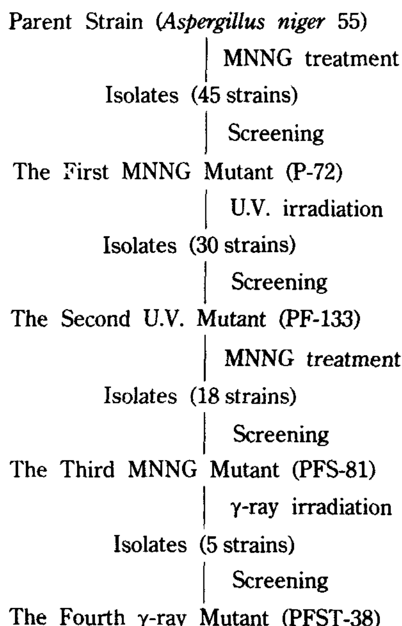

Fig. 2. Improvement of *Asp. niger* 55 strain with successive mutation.

Table 1. Morphological characteristics of the mutants

Strain	Conidia		Conidiophore			Conidial heads	
	Shape	Diameter (μm)	Color	Length (mm)	Diameter (μm)	Shape	Diameter (μm)
55 (Parent)	G	3.0~5.0	B	1.0~1.5	5~20	G	200~300
P-72	G	2.0~3.5	B	0.7~1.2	4~15	G	100~150
PF-133	G	2.0~3.0	B	0.7~1.0	4~13	G	80~120
PFS-81	SG	0.5~1.5	PB	0.3~0.5	2~ 6	G	30~ 80
PFST-138	SG	0.5~1.0	PB	0.1~0.3	2~ 6	SG	30~ 60

*Medium: Potato dextrose agar, Incubation temp.: 30℃, for 7 days.
*Symbol: G; globose, SG; subglobose, B; brown, PB; palebrown.

Table 2. Cultural characteristics of the mutants

Strain	Czapek's solution agar	Malt extract agar	Potato dextrose agar
55	++ DCB 5~7 cm	+++ DCB 7~8 cm	++ DB 6~7 cm
P-72	+ DCB 3~5 cm	++ CB 5~6 cm	+ B 4~5 cm
PF-133	+ DCB 3~4 cm	++ CB 5~6 cm	+ B 4~5 cm
PFS-81	± CB 2~3 cm	+ CB 3~4 cm	± PB 2~3 cm
PFST-38	± PCB 1~2 cm	+ CB 2~3 cm	± PB 1~2 cm

*Incubation temp.: 24~26℃, for 10 days.
*Growth: ± poor, + moderate, ++ good, +++ excellent.
*Color: PB; pale brown, PCB; pale chocolate brown, B; brown. CB; chocolate brown, DB; deep brown, DCB; deep chocolate brown.

productivity was selected and further exposed to combined mutagen treatments. As a result, 5 clones were isolated. Among them, one clone with significantly high glucoamylase and low transglucosidase activity was selected and designated as mutant PFST-38.

Morphological characteristics of the mutants

In order to learn whether morphological differences existed between the parental strain and the mutants derived from it, the shaped and sizes of conidia were examined. The results are shown in Table 1 and 2, and in Fig. 3. Inspecting the morphological and cultural characteristics of the various isolated mutants described above, it would seem that the diameter of conidia and conidiophore, the length of conidiophore, and the tendency to form conidia are closely correlated to the productivity of glucoamylase. The production of enzymes was found to be inversely proportional to the diameter of the conidia, the length of conidiophore, and the tendency to form conidia. The effects of temperature on the parent and its mutants are shown in Table 3. The conditions for growth revealed no marked differences between the parental strain and its mutants, but the characteristics of conidia for-

Fig. 3. Microphotographs of conidia of *Asp. niger* mutant PFST-38.
*The strain was grown on potato dextrose agar slant at 30℃ for 10 days.
*One scale indicates 10 μm.

Table 3. Effect of temperature on the growth and conidia formation of various mutants

Strain	10℃		20℃		30℃		40℃		50℃	
	MG	CF	MG	CF	MG	CF	MG	CF	MG	CF
55	+	−	++	+	++++	++	++	+	−	−
P-72	+	+	++	+	+++	+	++	+	−	−
PF-133	−	−	+	+	+++	+	++	+	−	−
PFS-81	−	−	+	+	++	+	+	+	−	−
PFST-38	−	−	+	−	++	+	+	−	−	−

*Symbol: MG; Mycelial growth, CF; Conidia formation, −; no growth, +; very poor growth, ++; poor growth, +++~++++; abundant growth.
*Medium: Potato dextrose agar slant, Incubation period; 7 days.

Table 4. *R* values of some mutants of *Asp. niger* 55

Strain	Diameter of agar block (cm)	Diameter of clearance (including the colony within, cm)	*R* Value
55	0.40	1.30	3.30
P-72	0.35	1.40	4.00
PF-133	0.35	1.45	4.14
PFS-81	0.25	1.55	6.20
PFST-38	0.20	1.60	8.00

*The ratio is the diameter of clearance to the diameter of the agar block.

mation were found to be different. The parental strain formed many conidia over a rather wide range of temperature, but the mutant strain formed only a few conidia and these only within a comparatively narrow range. The tendency to form conidia in the mutants decreased in accordance with the advancement of mutation treatment.

Comparison of enzyme productivity among the mutants

In order to ascertain the quantitative differences between the parental and mutant strains, measurements of RDA, glucoamylase, transglucosidase content were carried out (Table 4, Fig 4). From Table 5, it can be seen that mutant PFST-38 produced 11 times as much glucoamylase as the parental strain, No. 55. On the other hand, the productivity of transglucosidase was decreased by about 42% when compared with that of the mutant strain, PFS-81. Furthermore, the ratio of raw starch saccharifying activity to gelatinized starch saccharifying activity (RDA) of mutant PFST-38 was higher than that of the parental strain when corn meal was used as the substrate. These results suggest that mutant PFST-38 is one of the most promising producers of a raw starch saccharifying enzyme.

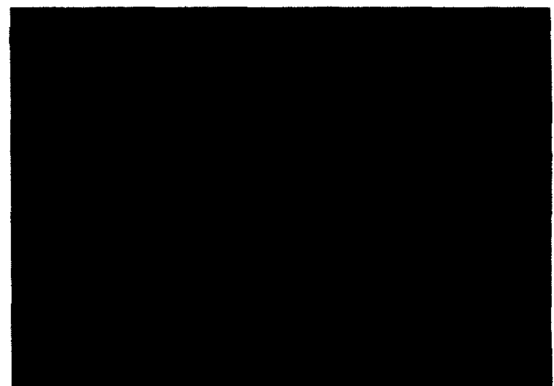

Fig. 4. Formation of clearing zone in the screening media cotained 2% raw corn meal powders.
*Symbols: 1, 55; 2, P-72; 3, PF-133; 4, PFS-81; 5, PFST-38.

Time courses of glucoamylase production in 30 *l*

Table 5. Comparison of enzyme productivity of various mutants

Strain	RDA (%)	Glucoamylase (μ/ml)*	Transglucosidase content**	TG/GA ratio***
55	91.0	5	9.0	0.18
P-72	93.0	11	12.1	0.11
PF-133	94.2	16	12.8	0.08
PFS-81	95.0	38	19.0	0.05
PFST-38	96.8	55	5.5	0.01

*Cultivation was carried out in 100 ml of the complete medium at 35℃ for 4 days using 500 ml Sakaguchi flask.
**Percent unfermentables formed from maltose.
***(Transglucosidase/Glucoamylase) Ratio is obtained by dividing the grams of unfermentable sugars synthesized per 100 gr. of maltose hydrate by 100 minus this value.

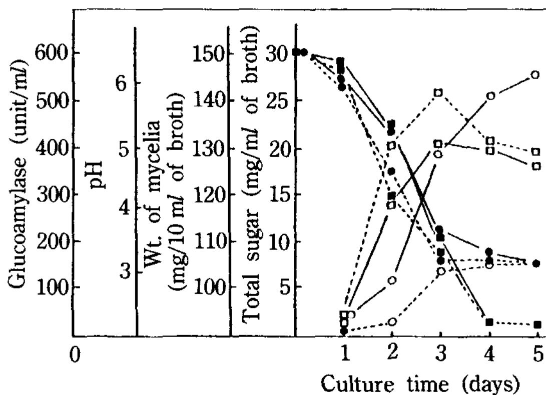

Fig. 5. Time courses of glucoamylase production of mutant PFST-38 and parent strain in liquid medium B.
*Symbols: ——, mutant PFST-38, ---, parent
○, Glucoamylase; ●, pH
□, Wt. of mycelia; ■, Total sugar
*Cultivation was carried out in liquid medium B at 30℃ for 5 days using 30 l fermentor.

Jar fermentor

The mutant PFST-38 was further tested for its ability to produce glucoamylase in the medium B. As shown in Fig. 5, in the case of mutant PFST-38, the peak of glucoamylase activity was observed at 120 hrs, whereas for the parent it was 96 hrs. Production of glucoamylase by mutant PFST-38 was 3.7 times that of the parent strain.

요 약

高 生產性 glucoamylase 活性을 가지면서 transglucosidase 活性이 거의 傷失된 變異株를 얻기 위해, 母菌인 *Aspergillus niger* 55에 MNNG 處理, U.V. 照射 및 γ-ray 照射를 하였다. 또한 동시에 變異株의 發生頻度를 높이기 위해 liquid culture-filtration과 conidia의 differential heat sensitivity를 利用하는 變異株濃縮 方法을 使用하였다. 그 結果, 母菌보다 glucoamylase 生產性이 11배 向上되고, transglucosidase 活性이 거의 傷失된 變異株 PFST-38을 얻었다. 變異株 PFST-38은 母菌과 形態的으로 比較할 때 conidia의 크기 및 形成 能力, conidiophore의 길이를 제외하고는 同一하였다. 以上의 結果로 볼때, 變異株 PFST-38은 液體 培養法에 의한 옥수수 生 澱粉 糖化 酵素의 生產에 매우 有用할 것으로 생각된다.

References

1. Oh, S.H. and P.S.O: *Kor. J. Appl. Microbiol. Bioeng.*, **18**, (1990)
2. Smiley, K.L., M.C. Cadmus, D.E. Hensley and A.A. Lagoda: *Appl. Microbiol.*, **12**, 455 (1964)
3. Cadmus, M.C., L.G. Jayko, D.E. Hensley, H. Gasdof and K.L. Smiley: *Cereal Chem.*, **43**, 658 (1966)
4. Smiley, K.L.: *Process for Obtaining Amyloglucosidase,* US Patent 3,301,768 (1967)
5. Park, Y.K. and M.S. Santi: *J. Ferment. Tech.*, **55**, 193 (1977)
6. Hayashida, S. and P.Q. Flor: *Agric. Biol. Chem.*, **45**, 2675 (1981)
7. Oh, S.H., H.J. Kwon and P.S.O: *Kor. J. Appl. Microbiol. Bioeng.*, **15**, (1987)
8. Bergmeyer, H. (ed.): *Methods of Enzymatic Analysis,* Academic Press. Inc. New York (1965)
9. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith: *Anal. Chem.*, **28**, 350 (1956)

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