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Cloning and expression of the α-amylase gene and oxytetracycline production in *Streptomyces rimosus*

Chun-Wen Cheng[1], Jin-Seng Lin[1], Yu-Tien Liu[2] and Shang-Shyng Yang[1,*]
[1]*Department of Agricultural Chemistry, National Taiwan University Taipei 10617, Taiwan*
[2]*Institute of Microbiology and Immunology, National Defense Medical Center, Taipei 10617, Taiwan*
**Author for correspondence: Tel.: +886-2-236 21 519, Fax: +886-2-236 79 827, E-mail: ssyang@ccms.ntu.edu.tw*

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Summary

An 8.4 kb *Sau3*AI DNA fragment containing the *Streptomyces rimosus* TM-55 α-amylase gene (*amy*) was ligated to a vector pIJ702, named pCYL01, and cloned into amylase deficient mutant *S. lividans* M2 (*amy*$^-$). Subcloning study showed that the *amy* gene was localized in 3.3 kb *Kpn*I-*Pst*I fragment. The molecular weight of the purified α-amylases of *S. lividans* M2/pCYL01 and *S. rimosus* TM-55 were estimated to be 65.7 kDa. Different sizes of recombinant plasmids carrying the *amy* gene had been retransferred into the parental strain of *S. rimosus* TM-55. Among these *S. rimosus* transformants, TM-55/pCYL01, TM-55/pCYL12 and TM-55/pCYL36 showed amylase activity 1.36- to 2.05-fold at the seventh day (1.61 to 2.42 units vs 1.18 units), and oxytetracycline (OTC) production 2.00- to 2.50-fold at the ninth day (approximate 140 to 170 μg ml^{-1} vs 72 μg ml^{-1}), higher than that of *S. rimosus* TM-55 alone, respectively. These results showed that industrial microorganisms could be improved by genetic and metabolic engineering.

Introduction

Streptomyces are important soil actinomycetes because of their ability to produce diverse secondary metabolites and are useful in biological and medical applications. Several genes encoding polysaccharide-degrading enzymes in *Streptomyces* species have been studied (Bahri & Ward 1993; Tsao *et al.* 1993). α-Amylase (EC 3.2.1.1), an extracellular enzyme, cleaves the α-1,4-glycosidic linkage in starch and generates shorter chains of oligosaccharide and even maltose. α-Amylase genes have been cloned and characterized, included *S. hygroscopicus* (Hoshiko *et al.* 1987), *S. lividans* (Tsao *et al.* 1993) and *S. venezuelae* (Virolle *et al.* 1988). *S. rimosus* TM-55 is an oxytetracycline (OTC)-producing strain. Our previous studies have shown that *S. rimosus* excretes high α-amylase activity into the medium during OTC production in submerged and solid-state fermentations (Wang & Yang 1995; Yang & Cheng 1996; Yang & Wang 1996, 1999). To investigate the application of the *amy* gene in OTC production, we cloned the *S. rimosus* TM-55 *amy* gene and retransferred the different sizes of recombinant plasmids harbouring the gene into *S. rimosus* TM-55. The α-amylase activity and OTC production in the parental strain of *S. rimosus* and its *amy* retransformants were also evaluated.

Materials and Methods

Test organisms

An amylase deficient mutant of *S. lividans* M2 (*amy*$^-$) used as a cloning host was obtained from Professor W.H. Hsu (National Chung-Hsing University, Taiwan). *S. rimosus* TM-55 (CCRC 940061) which expressed both amylase activity and OTC production was provided by Dr. Thomas H.H. Ku (Cyanamid Taiwan Corporation). *Bacillus subtilis* ATCC 6633 was purchased from the American Type Culture Collection. The plasmid pIJ702 was kindly provided by Professor D.A. Hopwood (John Innes Institute, UK) which has two selected markers of black pigment (*mel*) and thiostrepton resistance (*tsr*^{r}) genes.

Culture media and growth conditions

Streptomyces lividans M2 was cultivated in R2YE medium or YEME broth at 28 °C (Hopwood *et al.* 1985). The modified R2YE/starch medium replaced the sucrose with 1% (w/v) of soluble starch for secreening amylase activity. Yeast extract/starch (YS) medium containing (g l^{-1}) yeast extract, 5 and soluble starch, 10 was used for the expression of *Streptomyces* α-amylase. The medium used for antibiotic production contained (g l^{-1}): soluble starch, 20; corn steep liquor,

10; $(NH_4)_2SO_4$, 6; $CaCO_3$, 8; NaCl, 5 and soybean oil, 2 at pH 6.8 to 7.2. If necessary, 50 μg ml^{-1} of thiostrepton was also added for *Streptomyces* harbouring plasmid.

Isolation of chromosomal DNA and plasmids

Streptomyces mycelia were grown at 28 °C for 24 to 30 h and harvested by centrifugation. The cell pellet was resuspended in 2.5 ml STE buffer (containing 340 g sucrose l^{-1}, 25 mM Tris–HCl and 10 mM Na_2EDTA) with 2 mg lysozyme ml^{-1}, and followed by the addition of 10% (w v^{-1}) sarcosyl solution. The mixture was vortexed softly until it became clear. Afterwards, 9 g CsCl, 0.3 ml ethidium bromide and 0.5 ml TE buffer (containing 10 mM Tris–HCl and 1 mM EDTA at pH 8.0) were added to the previously lysed solution. The chromosomal DNA was centrifuged at 80,000 ×*g* for 48 h at 20 °C, and then the eluted DNA was extracted with *n*-butanol. Finally, the DNA was dialysed against TE buffer at 4 °C overnight.

DNA digestion, plasmid isolation, protoplast preparation and plasmid transformation of *Streptomyces* were performed according to the methods described by Hopwood *et al.* (1985).

Cloning of the α-amylase gene

Streptomyces rimosus TM-55 chromosomal DNA was partially digested with restriction enzyme *Sau*3AI. Six to 9 kb DNA fragments were collected from the 5 to 25% region of the NaCl linear gradient obtained after centrifugation at 68,000 ×*g* for 5 h at 25 °C. *Sau*3AI DNA fragment was compatibly ligated to the *Bgl*II-cleaved plasmid pIJ702 which had been pretreated with calf intestinal alkaline phosphatase (CIAP). The above *Sau*3AI genomic libraries were introduced into protoplasts of *S. lividans* M2 (*amy*$^-$). These transformants were cultivated on R2YE medium containing thiostrepton. All white (insertion inactivation of pIJ702 in *mel* gene) and thiostrepton-resistant (*tsr*^{r}) colonies were retransferred onto R2YE/starch medium by replica plating and incubated at 30 °C for 2 days. The plates were sprayed with iodine solution (0.127% I_2 and 1.66% KI in water), and the transformant with recombinant plasmid carrying an *amy* gene showed a clear zone. The host strain *S. lividans* M2 was used as a negative control in this screening. *Apa*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Kpn*I, *Hpa*I, *Hind*III, *Pst*I, *Sal*I, *Sac*I, *Sph*I and *Xho*I were used to construct the physical map of the recombinant pCYL01 with the *amy* gene from *S. rimosus* TM-55 as shown in Figure 1.

Purification of α-amylase

The α-amylase of the *S. lividans* M2/pCYL01 transformant was purified according to the procedures described in the previous paper (Yang & Cheng 1996). Mycelia were removed by centrifugation at 1500 ×*g* for 15 min. The supernatant was subjected to protein precipitation with ammonium sulphate between 30 and 70% of saturation. The precipitate was dissolved and dialysed in 20 mM phosphate buffer, pH 6.8 at 4 °C. It was further purified by gel filtration on a DEAE-Sephacryl column (1 cm i.d. × 28 cm) (Pharmarcia, Sweden) and eluted with 0 to 0.8 M of α-NaCl linear gradient in 0.02 M phosphate buffer at 0.14 ml min^{-1}. Fractions with enzyme activity were pooled and then passed through a Sephadex G-100 column (Pharmarcia, Sweden). Active fractions were concentrated with a Centricon microconcentrator (Amicon, USA).

α-Amylase activity assay

α-Amylase activity was measured as described by the Wilson & Ingledew (1982). One millilitre of enzyme solution and 2.0 ml of 0.2% soluble starch in 0.05 M K_2PO_4–NaOH buffer pH 6.0 were incubated at 40 °C for 10 min. The absorbance of reaction mixture 0.2 ml and iodine reagent (0.05% I_2 and 5% KI in water) 5.0 ml were measured at 620 nm against the blank. One unit of enzyme activity was defined as the amount of enzyme hydrolysing 1.0 mg of soluble starch in 10 min under the assay conditions.

Determination of oxytetracycline

OTC was determined by the paper disc method in antibiotic medium I (Merck No. 1 A-5272, Germany) with strain *Bacillus subtilis* ATCC 6633 at 30 °C (Yang & Ling 1989). Total OTC equivalent potency was calculated from the clear zone of a standard curve of OTC in the range of 1 μg ml^{-1} to 10 mg ml^{-1}. The regression study indicated that $Y = 0.1322X - 0.8917$ and $r^2 = 0.9912$, where Y is the log value of concentration of OTC, and X is the diameter of inhibition zone.

Chemical analysis

Total protein was determined by the Lowry method. Reducing sugar content was measured by the dinitrosalicylic acid (DNS) method.

Results

Cloning of an α-amylase gene from S. rimosus *TM-55*

About 3000 white (melanin defective) and thiostrepton-resistant *S. lividans* M2 transformants were collected. These colonies were transferred onto YS medium by replica plating, incubated for 48 h and then sprayed with I_2/KI solution. One of the *S. lividans* M2 transformants showed a clear zone around the colony under the purple-blue background. Plasmid was isolated from this colony and then tested for amylase activity. All of the *S. lividans* M2 retransformants showed amylase activity. The recombinant plasmid with the amylase activity was designated as pCYL01 (Figure 1).

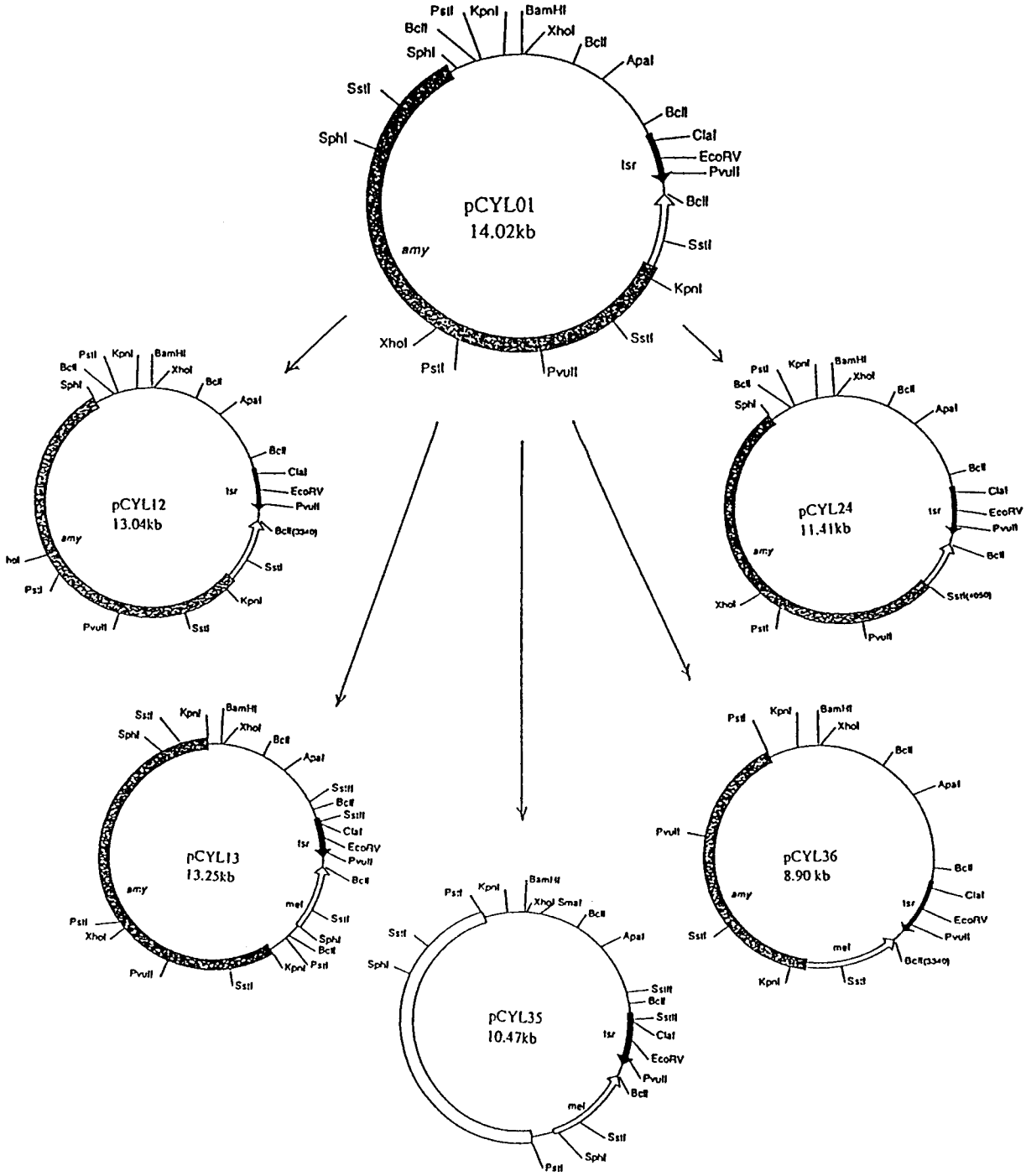

Figure 1. Subcloning strategies of the α-amylase gene from *S. rimosus* TM-55. Solid bar with *tsr* indicates thiostrepton-resistant; open bar with *mel* indicates tyrosinase and dot bar with *amy* indicates α-amylase, respectively. The recombinant plasmids of pCYL01, pCYL12, pCYL13 and pCYL36 showed α-amylase activity. pCYL24 showed weakly α-amylase and pCYL35 had no detectable amylase activity.

A series of subcloning and complementation experiments were performed with several restriction endonucleases. The DNA insert containing *Kpn*I to *Sph*I fragment had the α-amylase activity in *S. lividans* M2, included pCYL01, pCYL12 and pCYL13. The 1.84 kb *Kpn*I-*Sst*I deletion in pCYL24 showing weakly positive enzyme activity and no α-amylase activity was detected in the 2.87 kb *Kpn*I-*Pst*I deletion in pCYL35 (Figure 2). Finally, a plasmid with a 3.30 kb of *Kpn*I-*Pst*I fragment, named pCYL36, had the amylase activity as did the plasmid pCYL01 in *S. lividans* M2 (Figure 2). Thus, it can be concluded that the cloned *amy* gene from *S. rimosus* was located within this 3.3 kb insert.

Characterization of the α-amylase from S. rimosus

The biochemical properties of the α-amylase purified from the *amy* gene clone from *S. lividans* M2/pCYL01 were found to be same as *S. rimosus* TM-55 α-amylase. Purified α-amylase from both *S. lividans* M2/pCYL01

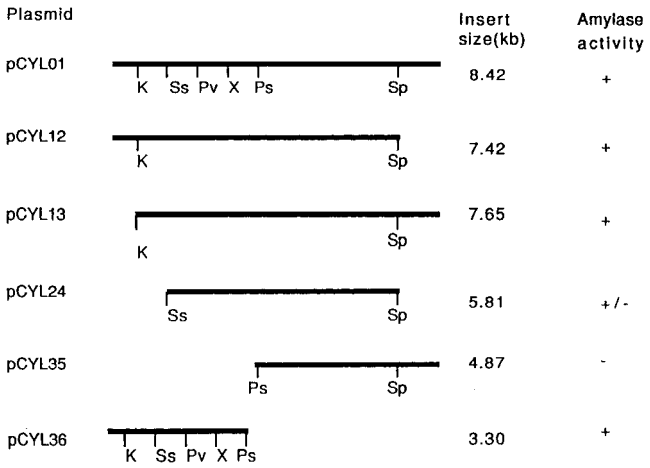

Figure 2. Restriction cleavage maps of the *S. rimosus amy* subclones. α-Amylase activities of the *S. lividans* M2 transformants harbouring each recombinant plasmid are indicated on the right side with positive (+) or negative (−). The solid bar represents the corresponding location of the DNA inserts. Abbreviations of enzyme cutting site represented as follows: K: *Kpn*I, Ss: *Sst*I, Pv: *Pvu*II, X: *Xho*I, Ps: *Pst*I, and Sp: *Sph*I.

and *S. rimosus* TM-55 showed the same electrophoretic pattern on SDS-PAGE. The molecular weight of *S. rimosus* TM-55 α-amylase was 65.7 kDa. Maltose and soluble starch were found to be better carbon sources than glucose for amylase production in the parental strain TM-55 as well as in its transformants carrying the *amy* gene. Starch concentration decreased with incubation time, whereas reducing sugar concentration decreased slightly on the first day, then increased gradually and reached the maximal value on the fifth to the seventh day. Starch consumption was found to be highest in *S. rimosus* TM-55/pCYL12 and *S. rimosus* TM-55/pCYL36, followed by *S. rimosus* TM-55/pCYL01, *S. rimosus* TM-55, and *S. rimosus* TM-55/PIJ702 respectively (Figure 3a). However, the reducing sugar concentration was maximal in *S. rimosus* TM-55/pCYL12 and *S. rimosus* TM-55/pCYL36, followed by *S. rimosus* TM-55/pCYL01, *S. rimosus* TM-55, and *S. rimosus* TM-55/pIJ702 had the lowest (Figure 3b). α-Amylase production was observed to be growth-associated in the medium containing 2% of carbon source. The optimal reaction pH values were between 5.5 and 6.5, and activity was stable at pH 6–8 for 24 h. Both activities had optimum reaction temperature between 37 and 40 °C. The enzyme activity was found to be about 40% when the reaction temperature was over 50 °C, and it was completely inactivated at 70 °C after 10 min incubation.

α-Amylase activity and oxytetracycline production

α-Amylase activities of *S. rimosus* TM-55/pCYL01, *S. rimosus* TM-55/pCYL36 and *S. rimosus* TM-55/pCYL12 were 1.36-, 1.44- and 2.05-fold higher than that of the parental strain *S. rimosus* TM-55 on the seventh day, respectively (Figure 4a). The enzyme activity in *S. rimosus* TM-55/pIJ702 was found to be same as that expressed in *S. rimosus* TM-55. α-Amylase activity of *S. lividans* M2 (*amy*$^-$) carrying pIJ702 was below 0.3 unit ml^{-1} and was used as a negative control.

The oxytetracycline production in the submerged fermentation is presented in Figure 4b. Transformants of *S. rimosus* TM-55/pCYL01, *S. rimosus* TM-55/pCYL12 and *S. rimosus* TM-55/pCYL36 had oxytetracycline potency about 2.00- to 2.50-fold higher than that of the parent strain *S. rimosus* TM-55 after 9 days of incubation. Oxytetracycline potency of *S. rimosus* TM-55/pIJ702 was almost undetected.

Discussion

The α-amylase gene of *S. rimosus* TM-55 localized in a 3.3 kb *Kpn*I-*Pst*I DNA fragment was cloned in

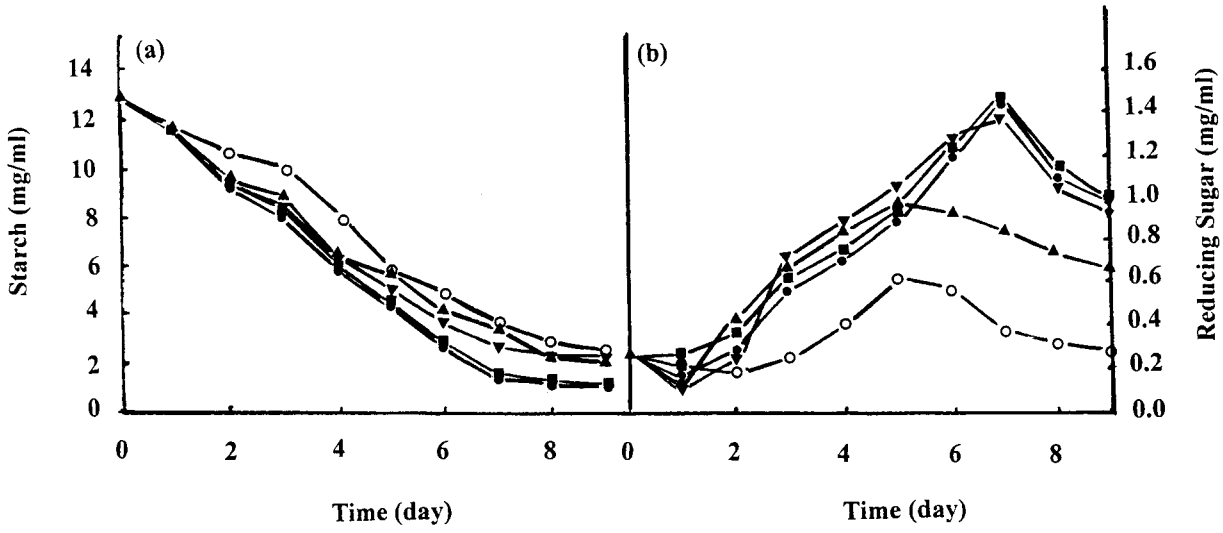

Figure 3. Concentration of starch and reducing sugar in *S. rimosus* TM-55 and its transformants. (a) Starch concentration, (b) reducing sugar concentration. ▲—▲, *S. rimosus* TM-55; ○—○, *S. rimosus* TM-55/pIJ702; ●—●, *S. rimosus* TM-55/pCYL01; ■—■, *S. rimosus* TM-55/pCYL12; and ▼—▼, *S. rimosus* TM-55/pCYL36.

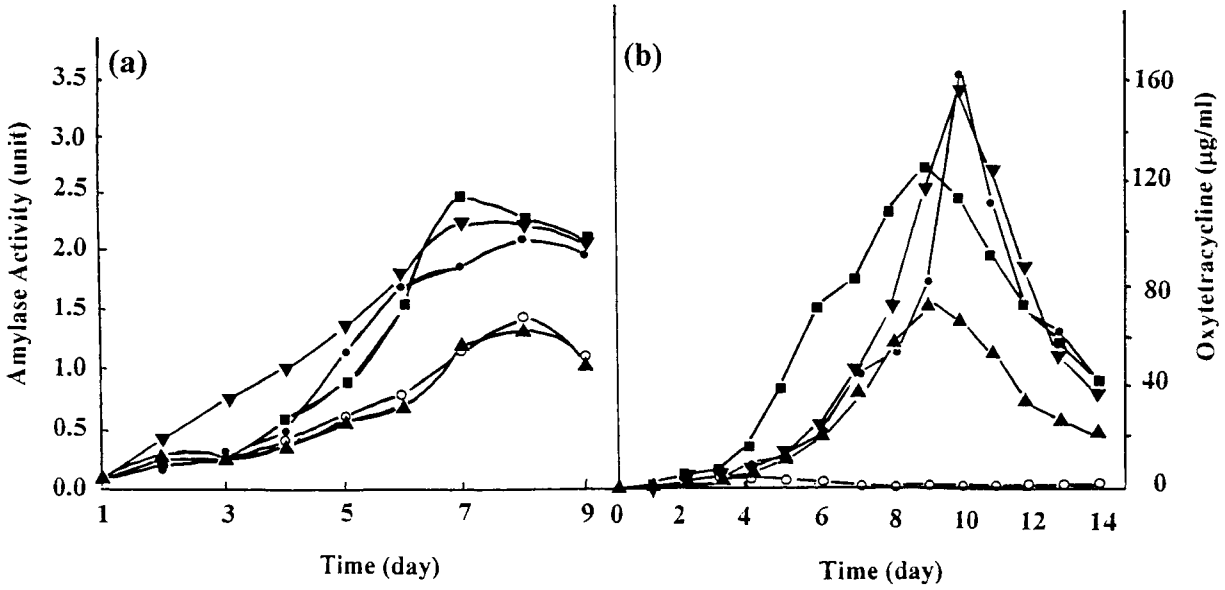

Figure 4. α-Amylase activity and oxytetracycline production in *S. rimosus* TM-55 and its transformants. (a) α-Amylase activity, (b) oxytetracycline production. ▲—▲, *S. rimosus* TM-55; ○—○, *S. rimosus* TM-55/pIJ702; ●—●, *S. rimosus* TM-55/pCYL01; ■—■, *S. rimosus* TM-55/pCYL12; and ▼—▼, *S. rimosus* TM-55/pCYL36.

S. lividans M2. The apparent molecular weight of purified α-amylase from *S. lividans* M2/pCYL01 harbouring *amy* was estimated by SDS-PAGE to be 65 kDa. This is close to the molecular weight of α-amylase of *S. limosus* (63 kDa) (Long *et al.* 1987) and somewhat larger than that of α-amylase of *S. griseus* (59.7 kDa) (Vigal *et al.* 1991; Garcia-Gonzalez *et al.* 1991), *S. hygroscopicus* (52 kDa) (Hoshiko *et al.* 1987), *S. thermoviolaceus* (49 kDa) (Bahri & Ward 1993) or *S. venezulae* (59 kDa) (Virolle *et al.* 1988), but smaller than that of α-amylase of *S. lividans* (100 kDa) (Tsao *et al.* 1993).

We have successfully retransferred the previously cloned plasmids carrying *amy* into *S. rimosus* TM-55. The length of pCYL01, pCYL12 and pCYL36 are different, but the transformants of *S. rimosus* TM-55 can express higher levels of α-amylase activity than that of the parental strain. It implied that the *amy* gene in *S. rimosus* TM-55 was amplified by using the high copy number of vector pIJ702. These transformants were also found to have higher OTC-producing potencies than that of *S. rimosus* TM-55. During cultivation, the starch concentration decreased and biomass and α-amylase activity increased after 7 days of incubation. In this study, we used starch as the sole source of carbon in submerged fermentation. *S. rimosus* TM-55 transformants harbouring *amy* could show both α-amylase activity and OTC production higher than that of their parent strain. Upon digestion of starch, the reducing sugar increased gradually. The presence of higher reducing sugar could thus be related to the higher α-amylase activity. Furthermore, the highest α-amylase activity in *S. rimosus* TM-55/pCYL12 may be due to the moderate length, among the three recombinant plasmids. *S. rimosus* TM-55/pCYL12 would have more *amy* gene products with consumption of the same nucleotide materials in stimulating α-amylase activity than that of pCYL01. Surprisingly, *S. rimosus* TM-55/pIJ702 could use soluble starch, however, its optimal reducing sugar content was about 35% lower than that of *S. rimosus* TM-55. The reason for the lower reducing sugar content in *S. rimosus* TM-55/pIJ702 cannot be explained at present and further investigation is needed. In addition, *S. rimosus* TM-55/pCYL01 and *S. rimosus* TM-55/pCYL36 had higher OTC potencies than that of *S. rimosus* TM-55/pCYL12 after 9-day incubation, and all of them had higher OTC production than that of *S. rimosus* TM-55. The correlation between α-amylase activity (growth-associated) and OTC production (secondary metabolite) in *S. rimosus* TM-55 is still unknown. Evidence has demonstrated that during the cultivation of *S. rimosus* TM-55, the OTC potency could be stimulated by maltose supplementation and inhibited by glucose addition (Yang & Yuan 1990). It was reported that the prevention of glucose repression and the reduction of energy charge could enhance the production of amylase and secondary metabolites (Demain 1972; Vining & Doull 1988; Cheng & Yang 1995). In solid state fermentation, maltose could enhance the production of tetracycline in *S. viridifaciens* and OTC in *S. rimosus*, respectively, whereas galactose or glucose inhibited the OTC production in *S. rimosus* (Yang & Ling 1989; Yang & Yuan 1990). These findings indicate that the production of enzymes and antibiotics can be regulated by different carbon sources.

Whether the production of OTC of these *S. rimosus* TM-55 transformants is affected by the percentages of starch, oligosaccharide and maltose which are produced by the cloned α-amylase secretion, or by any other factors in the medium (Demain 1989; Ali *et al.* 1993) during fermentation needs further study.

A novel finding is that no OTC titre was detected in the transformant of *S. rimosus* TM-55/pIJ702. It was known that melanin could be oxidized by tyrosinase, a monooxygenase, which is located on the intact pIJ702 (Hopwood *et al.* 1985). The functional group of OTC may be undergo deactivation by the tyrosinase (*mel*) oxidation of the transformant *S. rimosus* TM-55 carrying pIJ702 alone during OTC fermentation process. However, when we inserted the *amy* gene into the *mel* gene, the tyrosinase was found to be inactivated. Consequently, the OTC production in *S. rimosus* TM-55 transformants harbouring the *amy* gene could be prevented from oxidation by tyrosinase.

In summary, we report the cloning and characterization of the α-amylase gene from *S. rimosus* TM-55, and retransfer of this gene into its parental strain of *S. rimosus* TM-55. The *amy* transformants of *S. rimosus* TM-55 had both higher α-amylase activity and OTC production than those of the parental strain. Construction of such genetically and metabolically engineered microorganisms is going to gain much importance in industrial production of enzymes in the future.

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References

Ali, A.Z., Abdelrahman, N. & Baghlaf, A. 1993 Use of date products in production of oxytetracycline by *Streptomyces rimosus*. *Bioscience Biotechnology and Biochemistry* **57**, 987–988.

Bahri, S.M. & Ward, J.M. 1993 Sequence of the *Streptomyces thermoviolaceus* CUB74 α-amylase encoding gene and its transcription analysis in *Streptomyces lividans*. *Gene* **127**, 133–137.

Cheng, C.W. & Yang, S.S. 1995 Amylase production of *Streptomyces rimosus* TM-55 and their 2-deoxyglucose resistant mutants. *Chinese Journal of Microbiology and Immunology* **28**, 109–116.

Demain, A.L. 1972 Cellular and environmental factors affecting the synthesis and expression of metabolites. *Journal of Applied Chemistry and Biotechnology* **22**, 345–362.

Demain, A.L. 1989 Functions of secondary metabolites. In *Genetics and Molecular Biology of Industrial Microorganisms*, eds. Hershberger, C.L., Queener, S.W. & Hegeman, G. pp. 1–11. Washington D.C.: American Society for Microbiology. ISBN 1-55581010-1.

Garcia-Gonzalez, M.D., Martin, J.F., Vigal, T. & Liras, P. 1991 Characterization, expression on *Streptomyces lividans* and processing of the amylase of *Streptomyces griseus* IMRU 3570: two different amylases are derived from the same gene by an intracellular processing mechanism. *Journal of Bacteriology* **173**, 2451–2458.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. & Schrempf, H. 1985 *Genetic Manipulation of* Streptomyces: *a Laboratory Manual*. The John Innes Foundation, Norwich. ISBN 0-70840336-0.

Hoshiko, S., Makabe, O., Nojiri, C., Katsumata, K., Satoh, E. & Nagaoka, K. 1987 Molecular cloning and characterization of the *Streptomyces hygroscopicus* α- amylase gene. *Journal of Bacteriology* **169**, 1029–1036.

Long, C.M., Virolle, M.J., Chang, S.Y. & Chang, S. 1987 α-Amylase gene of *Streptomyces limosus*: Nucleotide sequence, expression motifs, and amino acid sequence homology to mammalian and invertebrate α-amylase. *Journal of Bacteriology* **169**, 5745–5754.

Tsao, L.S., Lin, L.L., Chen, J.C. & Hsu, W.H. 1993 Cloning and characterization of an α-amylase gene from *Streptomyces lividans*. *Biochimical et Biophysica Acta* **1171**, 255–262.

Vigal, T., Gell, J.A., Daza, A., Garcia-Gonzalez, M.D. & Martin, J.F. 1991 Cloning, characterization and expression of an α-amylase gene from *Streptomyces griseus* IMRU 3570. *Molecular Gene and Genetics* **225**, 278–288.

Vining, L.C. & Doull, J.L. 1988 Catabolite repression of secondary metabolism in *Actinomycetes*. In *Biology of Actinomycetes '88*. pp. 406–411. Tokyo: Japan Scientific Societies Press. ISBN 4-76221552-X.

Virolle, M.J., Long, C.M., Chang, S. & Bibb, M.J. 1988 Cloning, characterization and regulation of an α-amylase gene from *Streptomyces venezuelae*. *Gene* **74**, 321–334.

Wang, J.Y. & Yang, S.S. 1995 Morphogenesis, biomass and oxytetracycline production of *Streptomyces rimosus* in submerged cultivation. *Chinese Journal of Microbiology and Immunology* **28**, 21–31.

Wilson, J.J. & Ingledew, W.M. 1982 Isolation and characterization of *Schwanniomyces alluvius* amylolytic enzymes. *Applied and Environmental Microbiology* **44**, 301–307.

Yang, S.S. & Cheng, C.W. 1996 Production, purification and characterization of α-amylase by *Streptomyces rimosus*. *Journal of the Chinese Agricultural Chemical Society* **34**, 649–656.

Yang, S.S. & Ling, M.Y. 1989 Tetracycline production of sweet potato residue by solid state fermentation. *Biotechnology and Bioengineering* **33**, 1021–1028.

Yang, S.S. & Wang, J.Y. 1996 Morphogenesis, ATP content and oxytetracycline production by *Streptomyces rimosus* in solid substrate cultivation. *Journal of Applied Bacteriology* **80**, 545–550.

Yang, S.S. & Wang, J.Y. 1999 Protease and amylase production by *Streptomyces rimosus* in submerged and solid substrate cultivation. *Botanical Bulletin of Academia Sinica* **40**, 259–265.

Yang, S.S. & Yuan, S.S. 1990 Oxytetracycline production with sweet potato residue by solid state fermentation. *World Journal of Microbiology and Biotechnology* **6**, 236–244.