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Production of a novel FK520 analog in *Streptomyces hygroscopicus*: Improving titer while minimizing impurities

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FK520, also called ascomycin, is an immunosuppressive agent produced by *Streptomyces hygroscopicus*. Engineering the polyketide synthase genes of the parent strain generated novel FK520 analogs with the potential for improved *in vivo* stability. By replacing the acyl transferase (AT) domain in the polyketide synthase module 8 with an AT specific for methylmalonyl CoA (the rapamycin AT 3), the strain produced 13-desmethoxy-13-methyl-FK520 (13dmmFK520). Process development and scale-up studies of this recombinant *S. hygroscopicus* strain producing 13dmmFK520 are described here. Production kinetics and compound stability in fermentation broth were significantly different compared to the native FK520. Fermentation of the new strain resulted in the synthesis of a contaminating substance that co-purified with the 13dmmFK520. To optimize 13dmmFK520 production and to facilitate purification, growth parameters and media development were examined. Although a medium was identified that increased product titers by *ca.* 300%, the ratio of impurity to product was doubled. Lower dissolved oxygen (20% compared to 50% and 80%) increased titers by 20% with no appreciable effect on the concentration of impurity. Increasing the fermentation pH from 6.0 to 6.5 did not change the 13dmmFK520 titer, but reduced the impurity-to-product ratio by approximately 450%.
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Introduction

FK506 and FK520 are structurally related macrolide immunosuppressants produced by *Streptomyces* spp. FK506 is used in transplant patients to prevent organ rejection; however, dosing is difficult with FK506 since its metabolism varies from patient to patient. Studies indicate that metabolism of FK506 and FK520 occurs primarily in the liver and small intestine and is catalyzed by cytochrome *p*450 3A enzymes [3,5,9]. A primary target of the p450 enzymes on FK506 and FK520 is the 13-methoxy group. Due to the complexity of these molecules, the 13-methoxy group has not been accessible to synthetic chemists. Biotransformation has been employed to make novel FK506/520 analogs with modifications in the 13-position; however, these analogs contain a hydroxy group in that position, which is even less stable *in vivo* than the methoxy group [2]. Genetic approaches provide a means to access locations on macrolide polyketides that are not accessible by other means [1,4,8]. The FK506 and FK520 gene clusters are modular in nature [7,10] and amenable to such genetic manipulation.

It was hypothesized that replacement of the 13-methoxy group of FK520 with a hydrogen or methyl group may result in more stable compounds while retaining immunosuppressive activity. Using a genetic approach, 13-desmethoxy-13-methyl-FK520 (13dmmFK520) and 13-desmethoxy-FK520 analogs were generated by replacing the acyl transferase (AT) in module 8 of the FK520 PKS with the ATs from modules 3 and 12 from the rapamycin PKS, respectively (Reeves *et al*, manuscript in preparation). The structures of FK520 and 13dmmFK520 are shown in Figure 1.

The genetic approach to generating polyketide analogs as drug candidates is a new technology. Molecules derived this way are only now approaching clinical trials. Consequently, the impact of these genetic changes on process development is not well understood. For the genetic engineering described above, we found that production of 13dmmFK520 is reduced and delayed relative to production of FK520 in the parent organism. Also, purification of this analog was complicated by the production of an undefined impurity in relatively large quantities. Here we report on the development effort to improve the titer of 13dmmFK520 and limit production of this impurity. This paper defines key development studies that enabled production of 13dmmFK520 for structure confirmation, activity testing, and synthetic chemistry.

Materials and methods

Strains and culture conditions

Streptomyces hygroscopicus ATCC14891 and KOS60-135 were used for this work (Reeves *et al*, manuscript in preparation). Cell banks were prepared by adding glycerol (30% v/v final concentration) to a culture growing exponentially in medium 1 and then freezing 1-ml aliquots at −80°C. One cell bank vial was used to inoculate 50 ml of medium in a 250-ml baffled flask. The culture was allowed to grow for 2–3 days at 250 rpm and 30°C and then used to inoculate flask experiments at 5% of the final volume. For fermentation studies, the 50-ml culture was transferred to 500 ml of medium in a 2.8-l Fernbach flask, allowed to grow for 2 days, and used to inoculate 5 or 10 l of fermenters at 5% v/v.

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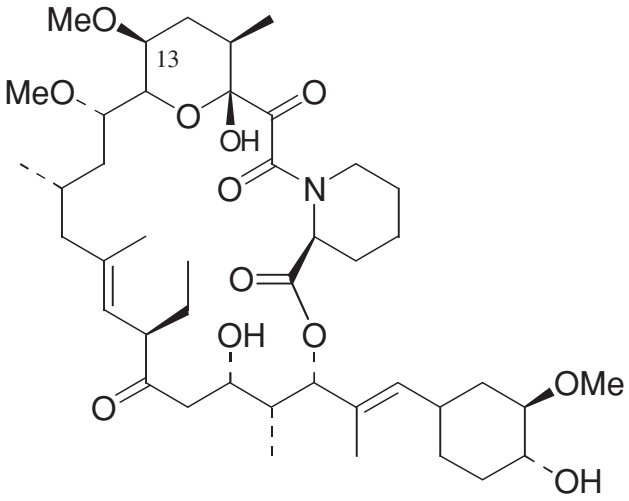
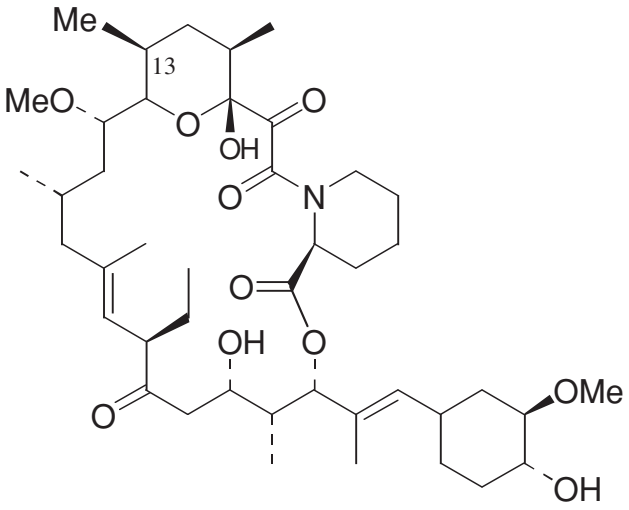


FK520



13-desmethoxy-13-methyl-FK520

Figure 1 Structure of FK520 and 13dmmFK520.

Media

Medium 1 was prepared by adding 30 g of tryptic soy broth (Difco, Sparks, MD, USA) per liter of deionized water and autoclaving the solution for 30 min at 121°C; after the solution had cooled, sterile glucose solution (500 g/l) was added to give a final concentration of 10 g/l. For flask studies, medium 1 included 100 mM MES buffer and was adjusted to pH 6.0. For fermentations in medium 1, seed and production cultures were grown in the same medium. Medium 2 and the associated seed medium are defined in Minas *et al* as F1 and V1, respectively [6].

Fermentation

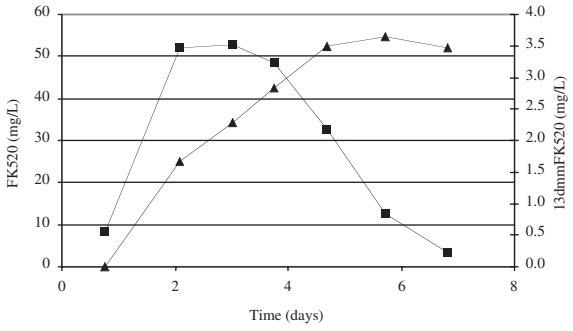
In 5- and 10-l fermenters (B. Braun Biostat MD and Biostat B, respectively), pH was controlled at 6.0, unless otherwise noted, by the addition of 2.5 N NaOH or 2.5 N H_2SO_4. The aeration rate was 0.4–0.8 VVM. Dissolved oxygen was controlled with agitation rate and oxygen enrichment to maintain the dissolved oxygen above 50% (except where noted). Agitation rate varied from 600 to 1000 rpm in the 5- and 10-l fermenters. Foam was controlled by the automatic addition of 50% antifoam B (JT Baker).

Inoculum cultures for 1000 l were started as described above in medium 1. Ten Fernbach cultures were combined and used to inoculate 100 l of medium in a 150-l fermenter (LSL Biolafitte, Allentown, PA, USA). The fermenter was controlled at 30°C, pH 6.0, by addition of 2.5–5.0 N H_2SO_4 and 2.5–5.0 N NaOH, and dissolved oxygen was maintained above 50% by agitation rate (50–600 rpm), air flow (10–50 l/min), and/or backpressure (0.1–0.3 bar). Foam was controlled by automatic addition of 50% (v/v) antifoam B. Growth continued in this reactor for 2 days.

The culture in the 150-l fermenter was used to inoculate 800 l of medium in a 1000-l fermenter (B. Braun Biostat UD). Foam, pH, and temperature were controlled as described above. Dissolved oxygen was maintained above 50% by agitation rate (150–300 rpm), air flow (100–600 l/min), and/or backpressure (0.1–0.4 bar).

Dry cell weight

For each time point, a 40-ml culture sample was centrifuged for 10 min at 3300×*g* in a preweighed 50-ml centrifuge tube. The supernatant was decanted and the cell pellet was washed with 40 ml of water. The sample was centrifuged again as described above. Water was removed and the cell pellet was placed in an 80°C oven for 24–48 h prior to taking a final weight. Dry cell weight is expressed as grams dry cell weight per liter of fermentation broth (g/l).



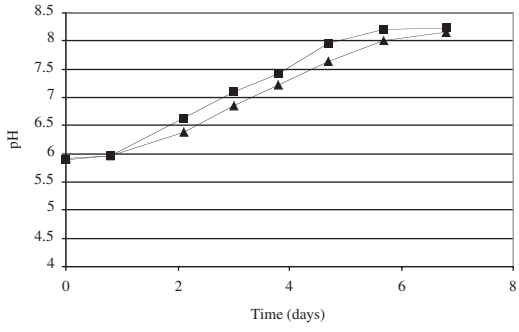


Figure 2 (A) Time course of FK520 (■) and 13dmmFK520 (▲) production (average titers, mg/l) in shake flask cultures grown in medium 1. (B) Symbols are the same for pH profiles.

Analysis of FK520 and 13dmmFK520

Identification of the 13dmmFK520 was made using LC/MS and NMR (Reeves *et al*, manuscript in preparation). Quantitation of the compound was carried out using a Hewlett Packard 1090 HPLC with UV detection at 210 nm. Whole broth samples for HPLC were extracted with an equal volume of methanol and centrifuged at 12,000×*g* to remove insoluble components. Supernatant (250 μl) was injected onto a 4.6×10-mm extraction column (Inertsil, C18 OD 53, 5 μm), washed with 50% acetonitrile for 2 min, and then eluted onto the main column (4.6×150 mm, same stationary phase and flow rate) with a 24-min gradient starting with 50% acetonitrile and ending with 100% acetonitrile. The 100% acetonitrile was maintained for 5 min. All solvents contained 5 mM acetic acid and the column was maintained at 50°C. With this method, FK520 eluted at 15 min and 13dmmFK520 eluted at 18 min. Standards were prepared using FK520 or 13dmmFK520 purified from fermentation broth. Error associated with this assay was approximately 10%.

Results and discussion

FK520 and 13dmmFK520 production

Shake flask studies were completed in duplicate to compare the production of FK520 and 13dmmFK520. Although both compounds are produced during the idiophase, the production rates and degradation rates of FK520 and 13dmmFK520 are different (Figure 2). The maximum production rates in medium 1 for FK520 and the FK520 analog were 44 and 1.3 mg/l/day, respectively. The maximum titer of 13dmmFK520 in medium 1 was 3.5–4.0 mg/l, 6–7% of the maximum titer of FK520. Based on our experience with AT swaps in other PKS genes, this initial decrease in production rate and titer is not surprising. The 13dmmFK520 compound was more stable than FK520 during fermentation (Figure 2), allowing for an extended production period in the same medium (5 days compared to 1 day).

The process in medium 1 was scaled to 10-l fermenters. In fermenters, the extended production period resulted in accumulation of a contaminating compound that was not produced in flasks. The contaminant eluted near the 13dmmFK520 peak, at 19.6 min in the HPLC protocol described in the Materials and Methods section (Figure 3). In 13dmmFK520-producing cultures, the analog proved extremely difficult to purify because of the relatively large quantity and hydrophobic nature of this impurity. Preliminary LC/MS and NMR indicated that the impurity was unrelated to FK520 so its structure was not completely elucidated.

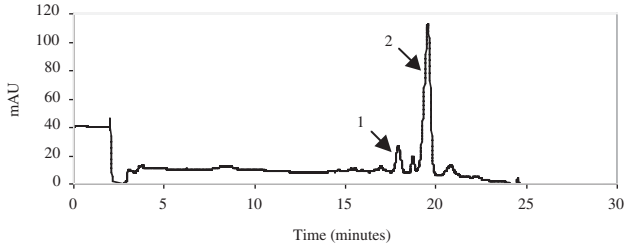


Figure 3 Representative HPLC chromatogram of extracted whole broth from a 10-l fermentation in medium 1 of KOS60-135 at pH 6.0. Arrow 1 indicates 13dmmFK520 eluting at 18 min. The major impurity elutes at 19–20 min (indicated by arrow 2).

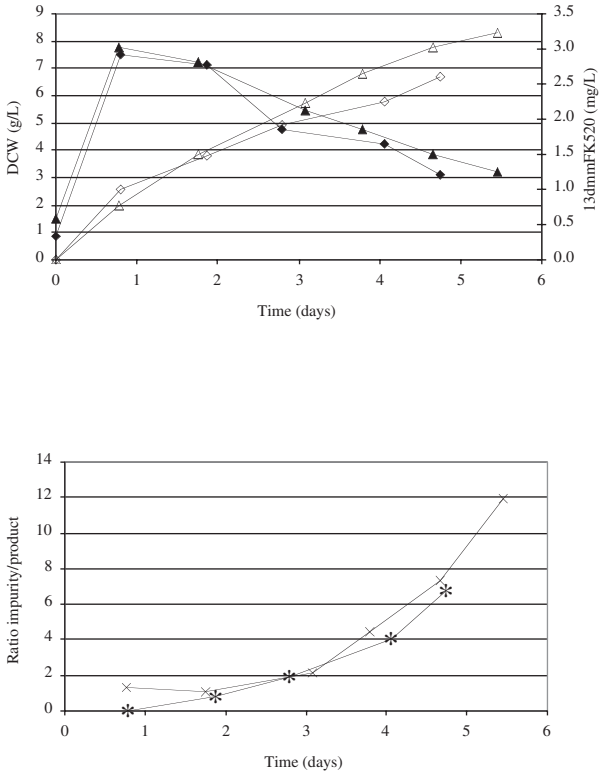


Figure 4 (A) Dry cell weight (g/l) and 13dmmFK520 titer (mg/l) of two 1000-l fermentations in medium 1. (▲) Dry cell weight run 1. (◆) Dry cell weight run 2. (△) 13dmmFK520 run 1. (◇) 13dmmFK520 run 2. (B) Impurity production (ratio of impurity UV peak area to product UV peak area) for runs 1 and 2. (×) impurity production run 1. (*) Impurity production run 2. Run 2 was harvested 1 day earlier than run 1.

In the case of FK520, the contaminating compound does not represent a purification obstacle because the culture is harvested prior to impurity synthesis.

Scale-up of 13dmmFK520 production and reduction of impurity

In an effort to produce material for structure and activity studies, 1000-l fermentations were performed early in the development process using medium 1. Figure 4A shows the growth of the organism for two consecutive runs. In medium 1, the maximum specific growth rate (μ_{max}) of this organism was 2.45 day^{-1} (doubling time=0.28 days). Growth occurs in the first day and cell density decreases after day 1. This gradual decrease is observed in both dry cell weight measurements as well as in the observed viscosity of the fermentation broth, and is consistent with growth in flasks. The onset of stationary phase coincides with the exhaustion of glucose (data not shown).

Figure 4A also depicts the production curves for the two 1000-l fermentations. Production of the FK520 analog starts within the first 24 h and continues through day 5. Production of the contaminating molecule occurs later in the fermentation (Figure 4B). As a strategy to decrease the amount of impurity produced while maximizing the 13dmmFK520 titer, a subsequent 1000-l run was terminated 1 day earlier than the first run. As shown in Figure 4, this strategy resulted in 45% of the impurity observed in the first run (based on UV peak area) while yielding 81% of the

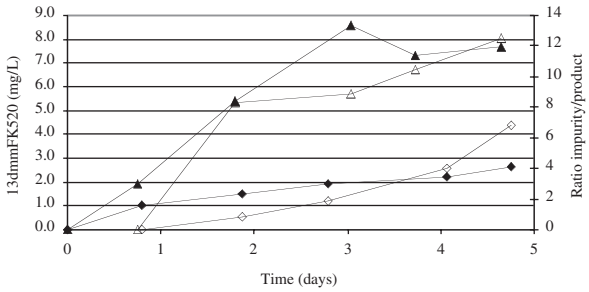


Figure 5 13dmmFK520 and impurity production with medium 1 and 2 in 1000-l fermentations. (◆) Product titer medium 1. (◇) Ratio of impurity to product medium 1. (▲) Product titer medium 2. (△) Ratio of impurity to product medium 2.

FK520 analog. Medium development and fermentation operating parameters were investigated in an effort to develop a consistent process with higher 13dmmFK520 yields and lower impurity.

Medium selection

Medium 1 was used for strain engineering and initial process development studies. A number of alternate media were screened for higher production of 13dmmFK520 (data not shown). Of the media tested, medium 2 resulted in titers that were approximately threefold higher than that of medium 1 (Figure 5). However, medium 2 presented other challenges. The absolute level of impurity was higher in medium 2, as well as the ratio of impurity to product. The ratio of impurity to product for medium 1 was 6.8 and the ratio of impurity to product for medium 2 was 12.5 (Figure 5). It was also observed that a larger percentage of the product was cell-associated in medium 2 (>90% as compared to 75% in medium 1). This percentage remained relatively constant during the fermentation process.

The effect of dissolved oxygen

In Medium 2, titers in the 5-l fermenters were about 25% higher than titers in the 1000-l fermenter (data not shown). The 5- and 1000-l fermentation processes were compared to explain the titer difference between them. The most noticeable operating difference between the bench top fermenters and the 1000-l fermenter was control of dissolved oxygen concentration. Dissolved oxygen was controlled in the 5- to 10-l fermenters using agitation. However, during the production period, the oxygen uptake rate exceeded the oxygen transfer rate and the dissolved oxygen decreased to 0% for approximately 1 day, starting 26 h after inoculation. The 1000-l fermenter used agitation, air flow, and backpressure to control dissolved oxygen and maintained a dissolved oxygen concentration >50% throughout the run. The laboratory reactors were glass and could not employ backpressure as a means to improve the K_La.

To determine the effect of dissolved oxygen concentration on 13dmmFK520 production in medium 2 at pH 6.0, dissolved oxygen was controlled in 5-l fermenters at 20%, 50%, and 80% of air saturation. To maintain these set points, dissolved oxygen was controlled with agitation, airflow, and oxygen enrichment when necessary. Although the time period of production was similar, the lowest dissolved oxygen value supported the maximum titer. This effect was apparent in duplicate runs with these oxygen set points. Figure 6 shows a representative experiment. Differences in growth rate or final cell density were not observed in these studies.

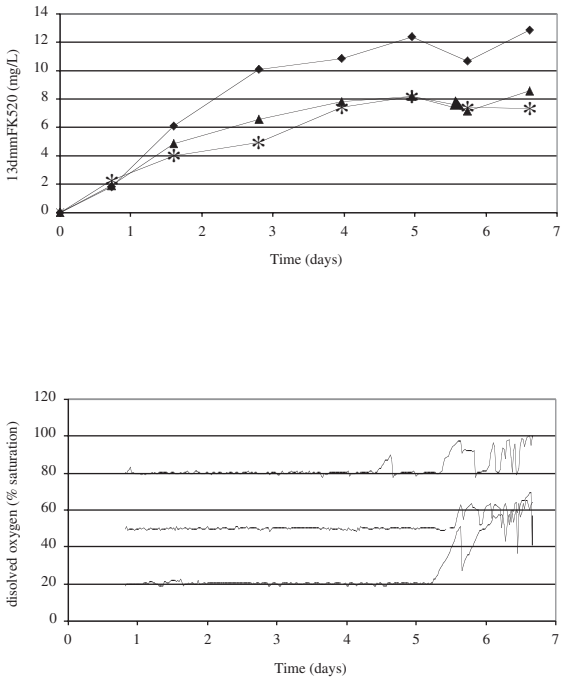


Figure 6 (A) Effect of dissolved oxygen concentration on 13dmmFK520 production in medium 2. Dissolved oxygen was maintained at 20% (◆), 50% (▲), and 80% (*) of air saturation. (B) Air saturation levels are shown without symbols.

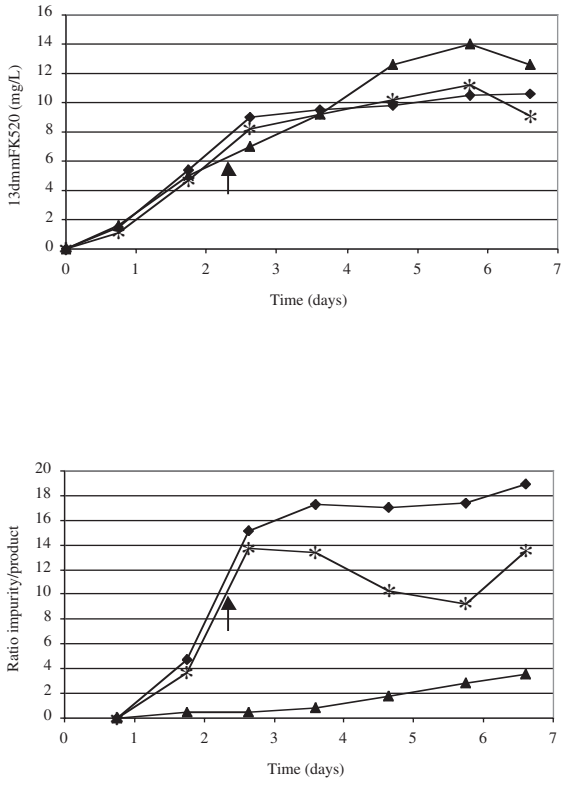


Figure 7 (A) Effect of pH on 13dmmFK520 production in medium 2. (◆) pH 6.0. (▲) pH 6.5. (*) pH 6.0 shifted to pH 7.0 where indicated with arrow. (B) Effect of pH on the ratio of impurity to product. Symbols for (A) and (B) are the same.

Dissolved oxygen changes did not have an appreciable effect on the final ratio of impurity to product.

The effect of pH

After medium 2 was identified and scale-up issues were resolved, development efforts shifted toward reducing the impurity concentration. Despite the MES buffer, the pH in flask cultures using medium 1 increased from 6 to 8 (Figure 2B), and flask fermentations generated only trace amounts of impurity. To determine if pH could be used as a tool to control impurity production in medium 2, an experiment was designed to duplicate the pH rise observed in flasks, and also to investigate higher pH throughout the run. Five-liter fermenters were run with pH controlled at 6.0, 6.5, and 6.0 with a shift to 7.0 after 2–3 days. Dissolved oxygen was held at 20% for all pH conditions. Increasing the pH from 6.0 to 7.0 between days 2 and 3 during the fermentation initially reduced the level of impurity; however, the final ratio of impurity to product was comparable to the fermenter controlled at pH 6 (Figure 7). The fermenter controlled at pH 6.5 had a dramatically lower concentration of impurity. A similar decrease in impurity production was obtained in a second set of fermenters run under these conditions (data not shown).

Overview

The expression of engineered polyketide synthases opens a new avenue for drug development. Because the technology for this approach is relatively young, the impact of genetic changes on process development is not well understood. Engineering ATCC 14891 (an FK520-producing strain) to make 13dmmFK520 changed production kinetics significantly. Product titers were lower and an undesirable byproduct was synthesized due to a longer production period. We successfully addressed these issues using traditional process development strategies. Production of 13dmmFK520 was increased threefold by changing the production medium and lowering the dissolved oxygen concentration from 80% to 20% during the fermentation. In addition, we found that changing the pH from 6.0 to 6.5 substantially reduced the ratio of impurity to product. The final process had threefold higher titer and a 50% lower ratio of impurity to product compared to the starting process. Further improvements with this recombinant organism may be obtained by random mutagenesis and selection, strain design, and medium development.

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