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(54) Title: AN IMPROVED FERMENTATION PROCESS FOR PREPARING ASCOMYCIN

(57) Abstract: The present invention relates to an improved fermentation process for the preparation of ascomycin.

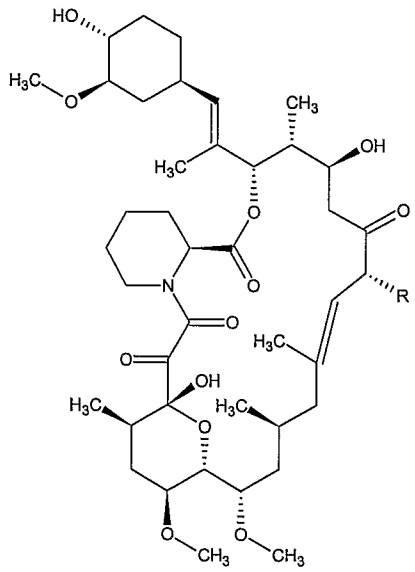
AN IMPROVED FERMENTATION PROCESS FOR PREPARING ASCOMYCIN

Field of the Invention

The present invention relates to an improved fermentation process for the preparation of ascomycin.

Background of the Invention

Ascomycin or FK-520, as exemplified in Formula I, is a well-known antifungal antibiotic, which inhibits filamentous fungi, for example, Penicillium chrysogenum and acid-fast bacteria. Ascomycin is also useful as a starting material for the synthesis of pimecrolimus, which is used for the treatment of atopic eczema.



FORMULA I

FK-520: R = $-CH_2CH_3$

FK-523: R = $-CH_3$

Ascomycin (FK-520) is usually produced by microbial fermentation, wherein during this fermentation process the homologous compound FK-523 is formed. Asocmycin (FK-520) must then be separated and purified from FK-523. The presence of FK-523 in ascomycin is undesirable as it has decreased immunosuppressive activity than ascomycin. FK-523 has a methyl group at C-21 resulting from the substitution of a propionate for a butyrate during polyketide synthesis. The separation and purification of ascomycin from the FK-523 compound produced during fermentation is often

complicated, the two compounds have almost the same number of carbon atoms and physical properties, such as solubility and affinity to solvents.

U.S. Patent No. 3,244,592 discloses the isolation of ascomycin from *Streptomyces hygroscopicus* subspecies *ascomyceticus* (ATCC 14891, MA 6475) and its use as an antifungal antibiotic.

U.S. Patent No. 4,894,366 discloses a method for the production of ascomycin and FK-523 by fermentation using *Streptomyces* subspecies *yakushimaensis* No. 7238 (FERM BP-928), wherein the ascomycin is isolated and purified from the fermentation broth by conventional methods.

Hatanaka et al. J. Antibiotics 41, 1592-1601, (1988) disclose a method for the production of ascomycin (FK-520) as an immunosuppressant from *Streptomyces* subspecies *yakushimaensis* No. 7238 (FERM BP-928, MA6531) and its subsequent purification by extraction from the fermentation broth followed by a crystallization step.

Junker et al. Biotechnology and Bioengineering 59, 595-604, (1998) disclose a method for the production of ascomycin from Streptomyces hygroscopicus by valine feeding during fermentation. Ascomycin produced under valine-fed conditions is reportedly contaminated with about 3.5% of the FK-523 homolog.

Junker et al. Biotechnology and Bioengineering 60, 580-588, (1998) disclose a method for the production of ascomycin using a valine-overproducing Streptomyces hygroscopicus mutant (MA7040) using soyabean oil and ammonium sulphate to optimize secondary metabolite formation.

The prior art methods target two approaches to control the level of FK-523 homolog impurity in the ascomycin produced by the fermentation technique. The first approach is the separation of FK-523 impurity from ascomycin by selective extraction from the fermentation broth and purification by a crystallization step. The second approach is by valine feeding during fermentation or employment of a valine-overproducing Streptomyces strain. FK-523 levels of between 2% to12% are observed in the ascomycin depending upon the quantity of valine fed.

These additional recovery methods used to purify ascomycin do not lead to the complete selective removal of the FK-523 impurity. In addition there is a significant loss of ascomycin due to the close structural similarity between ascomycin and FK-523.

Valine feeding leads to a significant reduction in the yield of ascomycin in range of about 20%.

Summary of the Invention

In one general aspect there is provided a fermentation process for the production of ascomycin. The process includes incubating an ascomycin producing microorganism in a nutrient medium at a temperature of between about 24°C to about 32°C and agitating at a rate of between about 2.0 to about 4.0 m/s to produce ascomycin.

Embodiments of the process may include one or more of the following features. For example, the incubation temperature may be between about 26°C to about 30°C, or it may be 28°C.

The agitation rate may be between about 2.0 and about 2.8 m/s. For example, the agitation rate may be 2.4 m/s.

The ascomycin producing microorganism may be an ascomycin hyperproducer or mutant thereof. The ascomycin producing microorganism may also be a Streptomyces species or mutant thereof. For example, the ascomycin producing microorganism may be Streptomyces hygroscopicus subspecies ascomyceticus (ATCC 14891).

The ascomycin producing microorganism is grown in a nutrient medium. The nutrient medium may include one or more of soya bean meal, dextrose, dextrin, milk, cotton seed meal, casein enzyme hydrolysate, CaCl2 and CaCO3.

The ascomycin produced may include less than about 6.0% of the homologous ascomycin compound FK-523. For example, the ascomycin may include less than about 5.0% of the homologous ascomycin compound FK-523.

In another general aspect there is provided an ascomycin compound. The ascomycin compound includes less than about 6.0% of the homologous ascomycin compound FK-523.

Embodiments of the compound may include one or more of the following features. For example, the ascomycin may include less than about 5.0% of the homologous ascomycin compound FK-523.

In another general aspect there is provided a method of inhibiting the growth of filamentous fungi or acid-fast bacteria in a mammal in need thereof. The method includes

administering a pharmaceutical dosage form comprising an ascomycin compound wherein the ascomycin compound comprises less than about 6.0% of the homologous ascomycin compound FK-523.

Embodiments of the method may include one or more of the following features. For example, the ascomycin compound may include less than about 5.0% of the homologous ascomycin compound FK-532.

Detailed Description of the Invention

The present inventors have surprisingly found that ascomycin having significantly reduced levels of the FK-523 homolog impurity may be produced by altering certain physico-chemical parameters, such as incubation temperature and agitation rates during fermentation. The present process is cost effective, commercially scalable without compromising the overall yield of ascomycin. The present process offers a simpler means of controlling the FK-520 impurity during the fermentation stage itself and results in marked improvement over the prior art methods for the production of ascomycin by fermentation.

The present process uses the ascomycin hyper producer strain Streptomyces hygroscopicus subspecies ascomyceticus (ATCC 14891). It is to be understood that the production of ascomycin by the present process is not limited to the use of the particular microorganism described herein. The present invention also includes the use of any mutants, which are capable of producing ascomycin, including natural mutants and artificial mutants, which can be produced from ascomycin producing microorganisms or mutants thereof by conventional methods known in the art.

Table 1 represents a comparison between the prior art methods for production of ascomycin and the present invention with respect to the fermentation conditions and level of FK-523 contamination in ascomycin. The present inventors have surprisingly observed that during the fermentative production of ascomycin, FK-523 formation may be reduced from about 20% to 4% using an incubation temperature of from about 24°C to about 32°C and an agitation rate from about 2.0-4.0 m/s.

TABLE 1

S.No.	Reference	Fermentation conditions	Level of FK-523 relative to FK-520
1	Journal of Antibiotics, 41:1592-1601, 1988.	**1.** Agitation rate constant (RPM 250) **2.** Temperature 30°C	25%
2	Biotechnology & Bioengineering, 59: 595 – 604, 1998	**1.** Agitation rate varied in cascade mode with aeration rate based on dissolved oxygen levels. (RPM 100-350) **2.** Temperature 28°C	12% to 14%
		1. Valine feeding during fermentation. **2.** Agitation rate varied in cascade mode with aeration rate based on dissolved oxygen levels. (RPM 100-350). **3.** Temperature 28°C	2% to 12%
3	Present Invention	**1.** Agitation rate varied between 2.4-4.0 m/s **2.** Temperature 24°C	17.8%
		1. Agitation rate varied between 2.0-3.3 m/s **2.** Temperature 24°C	9.29%
		1. Agitation rate varied between 2.0-3.3 m/s **2.** Temperature 28°C	4.7%

A first aspect of the present invention provides a fermentation process for the production of ascomycin, wherein the process includes incubating ascomycin producing microorganism in a nutrient medium at a temperature of about 24°C -32°C and agitation rate of 2.0-4.0 m/s.

The ascomycin producing Streptomyces strain is incubated in a nutrient medium for between about 180 to about 240 hours at 24°C to 32°C and is agitated at a rate of between about 2.0 to about 4.0 m/s. The level of ascomycin and FK-523 homolog impurity in fermentation broth is determined using a slight modification of the HPLC method reported in Biotechnology and Bioengineering 59: 595-604, 1998. Ascomycin

may be isolated from the fermentation mixture either using the whole broth or using the mycelium and the filtrate obtained therefrom. The incubation temperature may also be maintained at between about 26°C to about 30°C and the agitation rate may be set at between about 2.0 to about 2.8 m/s. For example, the incubation temperature may be maintained at about 28°C and the agitation rate at about 2.4 m/s.

The following non-limiting examples further illustrate the process parameters for the fermentation of ascomycin.

EXAMPLE 1

Streptomyces hygroscopicus subspecies *ascomyceticus* mutant (ATCC 14891) was grown and maintained on yeast extract malt extract agar slants. The surface of the slant was scraped and used to inoculate a 250 ml shake flask containing 35 ml of a sterilized seed medium containing dextrose (1.0 g/L), dextrin (10 g/L), milk (2 g/L), cotton seed meal (2.5 g/L), casein enzyme hydrolysate (5 g/L), $MgSO_4.7H_2O$ (0.05 g/L), $FeSO_4.7H_2O$ (0.025 g/L), NaCl (0.5 g/L), $CaCl_2$ (0.02 g/L), $ZnSO_4.7H_2O$ (0.01 g/L), $MnSO_4.7H_2O$ (0.005 g/L) and phosphate buffer (2ml /L; pH 7.0). The pH of the seed medium was adjusted to 7.2. The flask was incubated for 48 hours at 28°C on a rotary shaker. An aliquot of the resulting seed culture was used to inoculate a shake flask containing presterilized production medium consisting of dextrin (140 g/L), cotton seed meal (14 g/L), soya bean meal (10 g/L), glycerol (10 g/L), soya peptone (10 g/L), polyethylene glycol (12.5 g/L), potassium phosphate (0.8 g/L), and calcium carbonate (1.5 g/L). The pH was adjusted to 7.2. The flask was incubated at 24°C on a rotary shaker.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK 523 content: 18% (by HPLC).

EXAMPLE 2

The strain propagation and inoculum preparation methods were same as described in Example 1. 10 ml of the inoculum obtained in Example 1 was added to a 10 L bioreactor containing seed medium described in Example 1, except that the milk powder and cottonseed meal concentration was 3 g/L each and additionally calcium carbonate (0.5 g/L) was added. The medium was sterilized at about 121°C for about 30 minutes. After

47 hours of incubation, the seed culture was used to inoculate 20 L production bioreactor containing dextrin (30 g/L), cottonseed meal (10 g/L), soya bean meal (10 g/L), glycerol (10 g/L), soya peptone (10 g/L), polyethylene glycol (12.5 g/L), potassium phosphate (0.8 g/L) and calcium carbonate (1.5 g/L). A dose of dextrin solution was fed during fermentation when the pH rose beyond 7.6-7.8. The fermentation was run at about 24°C. The agitation rate was increased to between about 3.2 to about 4.0 m/s during growth phase. The agitation rate was then decreased during the production stage to between about 2.4 to about 2.8 m/s.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK 523 content: 17.8% (by HPLC).

EXAMPLE 3

The strain propagation, inoculum preparation and initial fermentation batch conditions were the same as described in Example 1 and 2, except that the agitation rates during growth and production phase was between about 2.7 to about 3.3 m/s and between about 2.0 to about 2.4 m/s respectively.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK 523 content: 9.29% (by HPLC).

EXAMPLE 4

The procedure of Example 1 was followed for microorganism propagation and fermentation, except that the production shake flasks were incubated at about 28°C.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK523 content: 7%.

EXAMPLE 5

The procedure of Example 1 was followed for microorganism propagation and fermentation, except that the production shake flasks were incubated at 30°C.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK523 content: 4.8%.

EXAMPLE 6

The procedure of Example 1 was followed for microorganism propagation and fermentation, except that the production shake flasks were incubated at 32°C.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK523 content: 5.0%.

EXAMPLE 7

The strain propagation and inoculum preparation methods were same as described in Example 1. 100 ml of inoculum obtained in Example 1 was added to 100 L seed fermenter containing seed medium comprising the components as described in Example 2. After 48 hours of incubation, 40 L of seed was used to inoculate 400 L production medium comprising components as described in Example 2. The fermentation was run at 28°C. A dose of dextrin solution was fed during fermentation when pH rose beyond 7.6-7.8. The agitation rate during growth and production phase was between about 2.7 to about 3.3 m/s and between about 2.0 and about 2.4 m/s respectively.

The resulting yield was:

Ascomycin: 350-400 mg/L (by HPLC)

FK 523 content: 4.7% (by HPLC).

While the present invention has been described in terms of its specific embodiments, certain modifications and equivalents will be apparent to those skilled in the art and are included within the scope of the present invention. The examples are provided

to illustrate particular aspects of the disclosure and do not limit the scope of the present invention as defined by the claims.

We Claim:

1. A fermentation process for the production of ascomycin, wherein the process comprises incubating an ascomycin producing microorganism in a nutrient medium at a temperature of between about 24°C to about 32°C and agitating at a rate of between about 2.0 to about 4.0 m/s to produce ascomycin.

2. The process according to claim 1, wherein the incubation temperature is between about 26°C to about 30°C.

3. The process according to claim 2, wherein the incubation temperature is 28°C.

4. The process according to claim 1, wherein the agitation rate is between about 2.0 and about 2.8 m/s.

5. The process according to claim 4, wherein the agitation rate is 2.4 m/s.

6. The process according to claim 1, wherein the ascomycin producing microorganism is an ascomycin hyperproducer or mutant thereof.

7. The process according to claim 6, wherein the ascomycin producing microorganism is a Streptomyces species or mutant thereof.

8. The process according to claim 7, wherein the ascomycin producing microorganism is *Streptomyces hygroscopicus* subspecies *ascomyceticus* (ATCC 14891).

9. The process according to claim 1, wherein the nutrient medium comprises one or more of soya bean meal, dextrose, dextrin, milk, cotton seed meal, casein enzyme hydrolysate, $CaCl_2$ and $CaCO_3$.

10. The process according to claim 1, wherein the ascomycin comprises less than about 6.0% of the homologous ascomycin compound FK-523.

11. The process according to claim 10, wherein the ascomycin comprises less than about 5.0% of the homologous ascomycin compound FK-523.

12. An ascomycin compound comprising less than about 6.0% of the homologous ascomycin compound FK-523.

13. The ascomycin compound of claim 12, wherein the ascomycin comprises less than about 5.0% of the homologous ascomycin compound FK-523.

14. A method of inhibiting the growth of filamentous fungi or acid-fast bacteria in a mammal in need thereof, the method comprising administering a pharmaceutical dosage form comprising an ascomycin compound wherein the ascomycin compound comprises less than about 6.0% of the homologous ascomycin compound FK-523.

15. The method of claim 14, wherein the ascomycin compound comprises less than about 5.0% of the homologous ascomycin compound FK-532.