The present invention relates to improved process of producing high yield of polymyxin B by fermentation. The present invention in particular provides an optimized fermentation conditions and an efficient process for the purification of polymyxin B.
Title:
PROCESS FOR PRODUCTION AND PURIFICATION OF POLYMYXIN B SULFATE

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

The present application claims priority to Indian Provisional Patent Application No. 2474/MUM/2008, filed November 24, 2009, which is entirely incorporated herein by reference.

FIELD OF THE INVENTION:

The present invention relates to an improved process of production and purification of polymyxin B sulphate. The present invention in particular relates to the fermentative production of polymyxin B sulphate in high yields.

BACKGROUND OF THE INVENTION

The polymyxin antibiotics were widely used to treat patients with various gram-negative bacterial infections including meningitis (Haemophilus influenzae), and urinary tract infections (E. coli) during 1960s and 1970s. Polymyxin B is also widely used to treat septic shock caused by endotoxin (Schindler M, Osborn MJ. "Interaction of divalent cations and polymyxin B with lipopolysaccharide" Biochemistry, 1979 Oct 2; 18(20): 4425^430). However, due to reports of toxic effects on the kidney and the nervous system, polymyxins fell out of clinical use in the 1980s. More recently, however, the polymyxins have gained renewed appreciation, especially in treating multi-drug resistant gram-negative bacterial infections (M.E.Falafas and A. Michalapолос, (2006) "Polymyxins: old antibiotics are back," The Lancet, 367: 633-634).

The polymyxins are a complex mixture of closely related decapeptides obtained from cultures of various strains of Bacillus polymyxa and related species (J. Shoji, H. Hinoo, Y. Wakisaka, K. Koizumi, M. Mayama, S. Mitsuura. J. Antibiotics. 30 (1977) 1029-1034). There are five main polymyxins (A, B, D, E and M), although only polymyxins B and E (colistin) have been used in clinical practice. Polymyxins contain characteristic constituents such as α,γ-diaminobutyric acid, L-threonine, and a fatty acid, and differ by the presence or absence of additional amino acid as well as the nature of the fatty acid. See Figure 1.
Polymyxin B is a cyclic, branched decapeptide that binds to membrane phospholipids and thereby interferes with membrane function. Polymyxin B is subdivided into at least four types, polymyxin B1, B2, B3 and B4, which differ from each other only in the fatty acyl moiety: B1 contains 6-methyloctanoic acid, B2 has 6-methylheptanoic acid, B3 has octanoic acid and B4 has heptanoic acid.


UK patent GB 645 750 provides a process for preparing sulphate or B-naphthalene sulphonate salts of polymyxin B and E, which are precipitated as the free base by treating an aqueous solution of such a salt with an alkali, such as aqueous ammonia, at a temperature between 50 and 100°C. The precipitated polymyxin base is then quickly removed from the hot supernatant liquid, washed with hot water, and dried or converted to its hydrochloride or other desired compound. A theoretical yield of about 96.8% of polymyxin E was obtained.

UK patent GB991602 describes a purification process for polymyxin which comprises treating an aqueous solution of the polymyxin with a permanganate at pH below 8.0. The examples describe the treatment of solutions of polymyxin B hydrochloride and of the sulphates of polymyxins A, B and E.

UK patent GB658766 describes purification by recrystallisation of the precipitated polymyxin base with an alcohol containing 2-5 carbon atoms, and further recrystallization after charcoal treatment. UK Patent GB 647925 describes the recovery of polymyxin at pH 4.5 with one or more sulphated fatty alcohols or esters, or their mixtures with salts. The resulting precipitate is separated by filtration and is dried, acid precipitated, and then converted to the base again. The base is then treated with activated charcoal or fractional precipitation.

UK patent GB 65897 describes *Bacillus polymyxa* fermentation culture in a substantially neutral nutrient medium under aerobic conditions for 2-5 days at 20-30°C while aerating with 4-64 litres of air per hour per 8 litres of medium. Bacteria and suspended material are removed, and polymyxin is adsorbed from the medium using, e.g., activated carbon or charcoal. The application describes additional examples of the preparation of derivatives of polymyxin, by treating the free base or hydrochloride of polymyxin with formaldehyde, acetaldehyde or 4-nitrobenzaldehyde.

Chinese patent CN 1800201 provides a method for preparing polymyxin E, which employs a foam separation method to extract and separate the polymyxin E from the fermentation liquor.

Many questions remain unanswered about polymyxins. Since most of the research was carried out before the 1980s, the methods for evaluation of the antibiotic were not as
advanced as today. Therefore, a detailed investigation on the polymyxin production by Bacillus polymyxa is warranted.

Looking to the long felt need for an efficient process resulting in a high yield of polymyxin, the present invention has focused on production of polymyxin by fermentation. By doing so the inventors have been successful in producing a high yield of polymyxin, such as 3g polymyxin B sulfate per litre of fermentation medium. The present invention provides an efficient method of producing polymyxin for large scale production, and in particular provides optimal culture conditions for a high yield of polymyxin B.

OBJECT OF THE INVENTION

It is the object of the present invention to provide an efficient process for producing polymyxin by fermentation.

It is the object of the present invention to provide an efficient process for producing a high yield of polymyxins.

It is the object of the present invention to provide a process for producing polymyxin, which is feasible on a large scale production.

The present invention in particular aims at providing optimal culture conditions that would result in high yield of polymyxin.

It is the object of the present invention to optimize the nutritional parameters of polymyxin.

It is the object of the present invention to provide a process for the purification of polymyxin.

SUMMARY OF THE INVENTION

The present invention provides an improved process for the production of a high yield of polymyxin B sulphate by fermentation, using glucose and oatmeal to maintain the carbon and nitrogen. The present invention provides an efficient method of producing polymyxin for large scale production. The present invention in particular provides optimal culture conditions for a high yield of polymyxin B.
In one embodiment the present invention provides a purification method for polymyxin.

In one embodiment, the invention is an aqueous composition (i.e., fermentation media) for the production of polymyxin B, including polymyxin B sulfate, by fermentation from *Bacillus polymyxa*. In a related embodiment, the fermentation medium comprises about 2% ammonium sulphate, about 0.2% dipotassium hydrogen phosphate, about 0.05% magnesium sulphate, about 0.05% sodium chloride, about 0.001% ferrous sulphate, about 0.5% bakers yeast autolysate, about 1-2% glucose, and about 2-3% oatmeal. In yet further embodiments, the composition comprises 2% oatmeal and/or, in others, 2% glucose. In related embodiments the pH is adjusted to about pH 6 to 7. The pH may be adjusted with 25%liquid ammonia or 5N HCl.

In another embodiment, the invention is a method of producing polymyxin B sulfate. In one related embodiment, the method comprises:

(a) inoculating *Bacillus polymyxa* into a fermentation medium of about 2% ammonium sulphate, about 0.2% dipotassium hydrogen phosphate, about 0.05% magnesium sulphate, about 0.05% sodium chloride, about 0.001% ferrous sulphate, about 0.5% bakers yeast autolysate, about 1-2% glucose and about 2-3% oatmeal;

(b) growing *Bacillus polymyxa* in the fermentation medium at 30°C with agitation and aeration for 72 h at a constant pH of 6.5. pH may be adjusted by addition of either 25% liquid ammonia or 5N HCl, as needed.

In further related embodiments, the agitation is about 250 rpm agitation, and the aeration is about 1.2 w m aeration for 72 h. In some embodiments, the *Bacillus polymyxa* is *Bacillus polymyxa* ATCC 10401. In some embodiments, a yield of at least 3.0g of polymyxin B sulfate per liter of fermentation medium is obtained.

In further embodiments, the invention comprises a method of purification of polymyxin B sulfate from fermentation medium. In one embodiment, the method of purification comprises:

(i) removing bacteria and solids to obtain a clarified fermentation medium;

(ii) adding about 2% activated charcoal to the clarified fermentation medium and mixing;

(iii) isolating the activated charcoal;
eluting the polymyxin B sulfate from the activated charcoal with a mixture of ethanol: acidified water (pH 2.0) (30:70) at 50°C, 25 rpm for 2 h;

(v) concentrating the eluate; and

(vi) dialyzing.

In further related embodiments, the purification further involves the steps of precipitation in acetone and/or lyophilization. In one embodiment, the purity of polymyxin B sulfate is greater than 90%. In other embodiments the purity is greater than 92%.

The invention also encompasses uses. In one embodiment, the invention is the use of the fermentation medium or methods of the invention for the production of polymyxin B sulfate. In related embodiments, the polymyxin is further used for the production of a medicament.

In another embodiment, the invention is the medicament made by growing *Bacillus polymyxa* in the composition of the invention, or obtained by the method of the invention. In a related embodiment, the medicament is for the treatment of infection by Gram-negative bacteria, and/or for the treatment of septic shock caused by the lipopolysaccharide of Gram-negative bacteria.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, the inventions of which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Fig.1: Structure and composition of various polymyxins.

Fig. 2. Effect of varying oatmeal concentration on production of polymyxin B

**DETAILED DESCRIPTION OF THE INVENTION**

Definitions:
As used herein, "about" when used in the context of a given numerical limitation encompasses a range plus or minus 10% of that number.

The term "polymyxin B" includes polymyxin B sulphate, and is not limited to any specific variant, i.e., sulphate of polymyxin B1, B2, B3, or B4, or any specific form.

"Gram-negative bacteria" refers to the group of prokaryotes having an outer membrane.

"Lipopolysaccharide" refers to the material of that name that is a major constituent of the outer membrane of gram-negative bacteria.

"Oatmeal" as used herein refers to grounded oats used in media preparation.

"Baker's yeast autolysate" as used herein refers to Bakers' yeast pastes which has been autoclaved and used in media preparation.

"Bacillus polymyxa" is used herein without being limited to specific variants of that bacterial species. In one embodiment, Bacillus polymyxa ATCC 10401 is used.

The present invention provides a process for the preparation of polymyxin, which results in high yields and can be produced on a large scale.

The present invention provides optimized conditions for fermentation. The present invention provides the effect of various physiological and nutrient requirements on fermentation. The present invention has focused on the nutritional parameters such as the amount of glucose and oatmeal in the production medium. These parameters showed a significant effect on the production of polymyxin B. Maximum yields were obtained with a medium having glucose at 1-2 % (w/v), and oatmeal at 1-3 % (w/v).

The effect of replacing yeast extract with bakers yeast autolysate on the production of polymyxin B was also studied. Bakers yeast autolysate was found to be more suitable for optimal production of polymyxin B. By replacing the yeast extract with bakers yeast autolysate, the yield was increased by 2-fold.

Using 25% liquid ammonia for maintenance of pH at 6 to 7 was important for polymyxin B sulfate production. There was no polymyxin B sulfate production if pH was not controlled.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to
constitute preferred modes for its practice. However, those of skill in the art will, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: Bacteria and growth conditions:

*Bacillus polymyxa* ATCC 10401 was maintained on nutrient agar. For production of polymyxin B sulphate, a seed culture was grown in nutrient broth incubated at 30°C, agitated at 200 rpm for 24 h.

For bioreactor studies, the cells were grown in the production medium sterile production media containing (w/v) 2% ammonium sulphate, 0.2% dipotassium hydrogen phosphate anhydrous, 0.05% magnesium sulphate heptahydrate, 0.05% sodium chloride, 0.001% ferrous Sulphate heptahydrate, 0.5% bakers yeast autolysate, 1% glucose and 2% oatmeal. The fermentation was carried out in 1.5 L bioreactor (B.Braun) at 30°C, 250 rpm agitation, 1.2 w/m aeration for 72 h. The pH of the medium was maintained at 6.5 by addition of either 25% liquid ammonia or 5 N HCl.

EXAMPLE 2: Bioassay of polymyxin B sulphate

Polymyxin B sulphate produced during fermentation was monitored by an agar diffusion bioassay, using *Escherichia coli* as an indicator strain. The zone size obtained by Standard Polymyxin B sulphate (1mg/ml) was compared with that of the sample (1mg/ml) and the purity was determined.

EXAMPLE 3: Optimization studies

Physiological and nutritional parameters for maximum production of polymyxin B sulphate were optimized at shake-flask level in production medium. Nutritional parameters were optimized by varying the glucose and oatmeal concentrations. pH control for optimal production of polymyxin B sulphate was revealed by the bioassay. There was no production if pH was left uncontrolled. The maintenance of pH for production of polymyxin B sulphate was found to be very stringent and the bioassay was positive only if the pH was controlled at pH 6 to 7.
Nutritional parameters such as the amount of glucose and oatmeal in the production medium also had a significant effect on the production of polymyxin B sulphate. A medium having glucose at 2 % (w/v) and oatmeal at 2-3 % (w/v) concentration gave the maximum yield of polymyxin B sulphate. Yeast extract also had a significant effect on PMB production, as replacement of yeast extract with bakers yeast autolysate increased the PMB yield by two-fold.

EXAMPLE 4: Production of polymyxin B sulphate in high yields in optimized medium

Fermentation at 1.5 L scale in the optimized medium having 2 % glucose and 2 % oatmeal at 30°C, 250 rpm, 1.25 w m aeration and at pH 6.5 gave a yield of 2g/L in 72 h.

EXAMPLE 5: Production of polymyxin B sulphate in optimized medium and conditions

Bacillus polymyxa ATCC 10401 was maintained on nutrient agar. For production of polymyxin B sulphate 1.5 L scale, the seed culture was grown in nutrient broth. The culture was incubated at 30°C at 200 rpm for 24 h.

The production medium consisted of (w/v) 2% ammonium Sulphate, 0.2% dipotassium hydrogen phosphate anhydrous, 0.05% magnesium sulphate heptahydrate, 0.05% sodium carbonate anhydrous, 0.001% ferrous sulphate heptahydrate, 0.25% bakers yeast autolysate, 2% glucose and 2% oatmeal. The production medium (1350 ml) was inoculated with 10% (w/v) seed culture. The fermentation was carried out in 1.5 L bioreactor (B.Braun) at 30°C, 250 rpm agitation, and 1.2 vvm aeration for 72 h. The pH of the medium was maintained at 6.5 by addition of either 25% liquid ammonia or 5 N HCl. A yield of 3 g/L was obtained in 72 h.

EXAMPLE 6: Purification of polymyxin B sulphate

100 ml of fermentation broth having a polymyxin B sulfate at 3.0 g/L was treated with activated charcoal at 2 % (w/v) and kept on an orbital shaker at 175 rpm for 2 h. After 2h, the mixture was centrifuged to recover charcoal to which polymyxin B sulphate was bound. Polymyxin B sulphate was eluted from the charcoal with a mixture of ethanol:
acidified water (pH 2.0) (30:70) at 50°C, 25 rpm for 2 h. The eluate was concentrated to one tenth its volume in a rota vapor and neutralized. The concentrate was dialyzed using a membrane having a molecular weight cutoff of 1.2 kD (benzoylated dialysis tubing, Sigma - Aldrich, D7884-10FT) against distilled water, overnight, at room temperature. The dialyzed solution was lyophilized. The lyophilized powder was tested against *E. coli* in the bioassay. The bioassay is carried out at different stages during the purification process. The lyophilized polymyxin B sulphate powder was 92% pure according to the bioassay, with a final yield of 35%.

**EXAMPLE 7**

Fermentation broth (100 ml) having a polymyxin B titre of 3.0 g/L was treated as per Example 5. The final yield was 99 mg of lyophilized polymyxin B sulphate powder with purity of 93.3% according to the bioassay, with a yield of 33%. A typical purification chart is shown below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (g/L)</th>
<th>Volume (mL)</th>
<th>Total concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before purification</td>
<td>3.0</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>After charcoal treatment</td>
<td>1.5</td>
<td>70</td>
<td>105</td>
</tr>
<tr>
<td>After dialysis</td>
<td>1.5</td>
<td>70</td>
<td>105</td>
</tr>
<tr>
<td>Lyophilized powder</td>
<td></td>
<td></td>
<td>99</td>
</tr>
</tbody>
</table>

**EXAMPLE 8: Purification of polymyxin B sulphate by dialysis and acetone precipitation**

Fermentation broth (1000 ml) having a polymyxin B titre of 2.15 g/L was treated as per Example 5, up to the dialysis step. After dialysis, the solution was concentrated in a rota vapor to one fifth its volume, and was added drop wise to a beaker containing 10 volumes of acetone to precipitate the polymyxin B sulphate. The sticky precipitate was collected, dissolved in distilled water and lyophilized. The lyophilized powder was tested against *E. coli* in the bioassay. The final recovery was 450 mg of lyophilized
polymyxin B sulphate powder with purity of 93.3 % according to the bioassay, and a yield of 21 %.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (g/L)</th>
<th>Volume (mL)</th>
<th>Total concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before purification</td>
<td>2.145</td>
<td>1000</td>
<td>2145</td>
</tr>
<tr>
<td>After charcoal treatment</td>
<td>1.136</td>
<td>1000</td>
<td>1136</td>
</tr>
<tr>
<td>Before dialysis</td>
<td>6.77</td>
<td>140</td>
<td>947.9</td>
</tr>
<tr>
<td>After dialysis</td>
<td>5.83</td>
<td>160</td>
<td>933.3</td>
</tr>
<tr>
<td>Lyophilized powder</td>
<td></td>
<td></td>
<td>450</td>
</tr>
</tbody>
</table>

Thus, while we have described fundamental novel features of the invention, it will be understood that various omissions and substitutions and changes in the form and details may be possible without departing from the spirit of the invention. For example, it is expressly intended that all combinations of those elements and/or method steps, which perform substantially the same function in substantially the same way to achieve the same results, be within the scope of the invention.
WE CLAIM:

1. A fermentation medium for the production of polymyxin B by fermentation from *Bacillus polymyxa*, comprising: ammonium sulphate, dipotassium hydrogen phosphate, magnesium sulphate, sodium chloride, ferrous sulphate, about bakers yeast autolysate, about 1-2% glucose, and about 2-3% oatmeal.

2. The fermentation medium of claim 2, comprising: about 2% ammonium sulphate, about 0.2% dipotassium hydrogen phosphate, about 0.05% magnesium sulphate, about 0.05% sodium chloride, about 0.001% ferrous sulphate, about 0.5% bakers yeast autolysate, about 1-2% glucose, and about 2-3% oatmeal.

3. The fermentation medium of claim 2, comprising 2% oatmeal.

4. The fermentation medium of claim 2, comprising 2% glucose.

5. The fermentation medium of claim 2, wherein the pH of the fermentation medium is about 6-7.

6. The fermentation medium of claim 5, which is adjusted to pH 6-7 with liquid ammonia or HCl.

7. A method for producing polymyxin B sulfate, comprising:
   (a) inoculating *Bacillus polymyxa* into a fermentation medium comprising about 2% ammonium sulphate, about 0.2% dipotassium hydrogen phosphate, about 0.05% magnesium sulphate, about 0.05% sodium chloride, about 0.001% ferrous sulphate, about 0.5% bakers yeast autolysate, about 1-2% glucose and about 2-3% oatmeal;
   (b) growing the *Bacillus polymyxa* in the fermentation medium at 30°C with agitation and aeration for 72 h at a constant pH of about 6-7.

8. The method of claim 7, wherein the pH is maintained by addition of either 25% liquid ammonia or 5 N HCl.

9. The method of claim 7, wherein the agitation is about 250 rpm agitation, and the aeration is about 1.2 w m aeration for 72 h.

10. The method of claim 7, wherein the *Bacillus polymyxa* is *Bacillus polymyxa* ATCC 10401.

11. The method of claim 5, wherein the method produces a yield of at least 3.0g per liter of L of fermentation medium.
12. A method of purification of polymyxin B sulfate from fermentation medium, comprising:

(i) removal of bacteria and solids from the fermentation medium to obtain a clarified medium;

(ii) adding 2% activated charcoal to the clarified medium, and mixing;

(iii) isolating the activated charcoal;

(iv) eluting the polymyxin B sulfate with a mixture of ethanol: acidified water (pH 2.0) (30:70);

(v) concentrating the elute; and

(vi) dialyzing.

13. The method of claim 12, further comprising precipitation in acetone.

14. The method of claim 12, further comprising lyophilization.

15. The method of claim 12, wherein the purity of polymyxin B sulfate is greater than 90%.

16. The method of claim 15, wherein the purity is at least 93%.

17. Use of the fermentation medium of any of claims 1-6, for production of polymyxin B sulfate.

18. Use of the polymyxin B produced by any of claims 7-11 for the production of a medicament.

19. A medicament comprising the polymyxin B made by growing *Bacillus polymyxa* in the fermentation medium of any of claims 1-6, or obtained by the method of any of claims 7-16.

20. The medicament of claim 19, for the treatment of infection by Gram-negative bacteria.


22. A method for producing polymyxin B sulfate and its use, as claimed above exemplified herein substantially in the examples and figures.
Fig. 1 Structure and composition of various polymyxins

<table>
<thead>
<tr>
<th></th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin A</td>
<td>D-Dab</td>
<td>Leu</td>
<td>Thr</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>L-Dab</td>
<td>Phe</td>
<td>Leu</td>
</tr>
<tr>
<td>Polymyxin D</td>
<td>D-Ser</td>
<td>Leu</td>
<td>Thr</td>
</tr>
<tr>
<td>Polymyxin E</td>
<td>L-Dab</td>
<td>Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>Polymyxin M</td>
<td>L-Dab</td>
<td>Leu</td>
<td>Thr</td>
</tr>
</tbody>
</table>

Figure 2: Effect of varying oatmeal concentration on production of polymyxin B