Evaluation of *Bacillus polymyxa* ATCC 10401 for Polymyxin B Sulphate Production: Present Yield and Approaches for Industrial Feasibility

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ABSTRACT

The lyophilized culture of Bacillus polymyxa ATCC 10401 demonstrates a strong capacity to produce polymyxin B sulfate, a vital cationic antimicrobial peptide that is increasingly used in the management of multidrug-resistant Gram-negative illnesses. We examined the extent of cultural growth using a wheat flour-based medium hydrolyzed with α-amylase to enhance nutrient availability. Optimizing the medium in the shake flask helped the culture develop quickly and create antibiotics. The modified conditions were successfully applied to a 100L stainless steel semi-automatic fermenter, where the colony continued to thrive. When fermentation was carefully controlled, the maximum titer reached was 1.2 g/L. Downstream processing, which comprised separating cells, correcting the pH, purifying, and crystallizing, made polymyxin B sulfate in pure powdered form with a recovery rate of roughly 70%. These findings indicate that Bacillus polymyxa ATCC 10401 is an effective producer of polymyxin B sulfate, and that agro-industrial substrates can be utilized for large-scale, cost-effective production. However, more improvements to the culture strain and media composition are needed to produce higher titers that are good for large-scale industrial production.

KEYWORDS:

Polymyxin B sulfate, Bacillus polymyxa, lyophilized culture, multidrug-resistant Gram-negative infections, and non-ribosomal peptide synthetases (NRPSs).

1. INTRODUCTION

Polymyxin B sulfate is a strong cationic antibacterial peptide that belongs to the polymyxin family. It is primarily utilized as a last-resort therapy for multidrug-resistant Gram-negative bacteria such as Pseudomonas aeruginosa, Acinetobacter baumannii, and Klebsiella pneumoniae [1–3]. The troubling rise in antibiotic resistance has led to a renewed emphasis on the efficient and cost-effective production of polymyxin B for medical and pharmaceutical purposes [4,5]. Polymyxin B is derived from the

Bacillus polymyxa species, and its production is contingent upon the strain's physiological characteristics and the composition of the fermentation medium [6,7]. Non-ribosomal peptide synthetases (NRPSs) are part of the process.

In this context, Bacillus polymyxa ATCC 10401, a lyophilized culture with documented biosynthetic capabilities, emerges as a promising candidate for the synthesis of polymyxin B sulfate [8]. But the production titers that were given still make it challenging to scale up for application in industry. This means that both the culture conditions and the medium components need to be examined and modified very carefully [9]. Using agro-industrial substrates like wheat flour and enzymatic hydrolysis to make nutrients more available is a cheaper way to do things than using typical complicated media [10,11]. Also, to acquire the most metabolites, you need to pay close attention to things like pH, temperature, aeration, and agitation while the fermentation process is going on [12–14].

The purpose of this study is to determine the efficacy of B. can produce polymyxin B sulfate. Polymyxa ATCC 10401 in the best conditions for shaking flasks and fermenters. It also looks at how well a medium made from wheat flour works and demonstrates the greatest titer that has been attained so far. Although the technique performed well when it was scaled up to a 100L fermenter and downstream recovery, the results reveal that the strain's performance and media formulation need to be enhanced much more in order to get titers that are good for industrial-scale production [15]. The results give us useful information that will help us design a cheap way to make polymyxin B on a large scale.

The purpose of this experiment was to find a cheap and easy approach to produce polymyxin B sulfate, starting with optimizing medium in shaking flasks and working up to a 100L stainless steel fermenter. Using a medium produced from hydrolyzed wheat flour, the culture obtained a maximum titer of 1.2 g/L. This was successfully replicated during scale-up in regulated conditions.

2. MATERIALS AND METHODS

2.1. Culture Activation and Preservation:

The American Type Culture Collection (ATCC, USA) sent us the lyophilized strain of Bacillus polymyxa ATCC 10401. To bring the culture back to life, it was aseptically reconstituted in sterile Lab Inoculum (LI) media and kept at 30 ± 1 °C for 24 hours without moving it. Then, a loopful of the activated culture was distributed on nutrient agar slants and maintained at 30 ± 1 °C for 24 to 48 hours to get dense colony formation [8]. Every two weeks, well-established slants were sub-cultured and kept at 4 °C to make working stocks. To maintain the cultures alive for future studies, fully grown slants were stored at 4 °C for a long time.

2.2. Preparation of Inoculum:

The first culture was begun in 25 mL of sterilized media with the following concentrations (g/L): Glucose – 30.0, Peptone – 30.0, $MgSO_4 \cdot 7H_2O$ – 0.5, and K_2HPO_4 – 2.0. After sterilization, the pH was set at 6.40 ± 0.10. We introduced a 10% (v/v) inoculum from a recently revived culture to the medium

and shook the flasks at 250 rpm for 24 hours at 30°C. After incubation, the culture had a pH of 5.40 ± 0.40 and a packed cell volume (PCV) of 20 ± 05 g/L. This suggested that it was ready to move on to the seed culture stage [12].

2.3. Preparation of Seed Culture:

We began seed cultures in 250 mL conical flasks, each containing 25 mL of seed medium. It took 50.0 g of wheat flour, 1.0 g of amylase, 6.0 g of yeast extract, 5.0 g of ammonium sulfate, 6.0 g of $CaCO_3$, and 0.5 g of K_2HPO_4 to make the medium. At 90 °C for 30 minutes, amylase broke down the wheat flour. The pH of the medium was 6.80 ± 0.10 before sterilization and 6.40 ± 0.10 after sterilization. The other parts were added after the medium had cooled down, and the pH stayed the same. We poured 10% (v/v) of the lab culture into flasks and stirred them at 250 rpm for 24 hours at 30°C. Microscopically, sterility was confirmed, and the culture reached a pH of 5.40 ± 0.40 and a packed cell volume (PCV) of 40 ± 05 g/L, showing that it was ready to move on to the next stage of production [12,14].

2.4. Production:

To make cultures for production, 25 mL of production media was put into each of the 250 mL conical flasks. There were 50.0 g of wheat flour, 1.0 g of amylase, 10.0 g of CSM, 0.50 g of K_2HPO_4, 5.0 g of ammonium sulfate, 8.0 g of $CaCO_3$, and 2.0 g of L-threonine in each liter of the medium. For 30 minutes at 90°C, amylase was used to break down wheat flour. After that, the additional parts were added. The pH was 6.80 ± 0.10 before sterilization and 6.40 ± 0.10 after sterilization. The flasks were shaken at 250 rpm for 48 to 72 hours at 30°C after adding 10% (v/v) of the seed culture. After 24 hours, the initial titre was 0.40 g/L, and after 72 hours, it was 1.02 g/L. The pH stayed between 5.50 and 0.50 for the whole incubation, and the packed cell volume (PCV) was 29 g/L at different intervals [10,11].

2.5. Fermenter-Based Inoculum and Seed Development:

The scale-up process was done in steps to make sure that the culture operated and lived. To begin, the laboratory inoculum (LI) was prepared in 250 mL flasks containing 25 mL of LI media and maintained at 30°C with agitation at 250 rpm for 24 hours. We mixed this culture with 500 mL of the same medium and put it in 5 L flasks (10% v/v) that were kept in the same conditions. The cultures had a pH of 5.40 ± 0.50 after being incubated, a packed cell volume (PCV) of 20 ± 05%, and a microscopic examination confirmed that they were sterile [13,14].

Then, 10% (v/v) of this seed culture was transplanted aseptically into a 20 L stainless steel fermenter that had the same seed media as the shake flasks. The fermenter worked at 30°C and added air at a rate of 0.7 vvm. The agitation was modified based on how much dissolved oxygen (DO) there was. When the DO levels fell below 30%, the tip speed went from 3.0 m/s to 4.0 m/s. The culture had a pH of 5.40 ± 0.50 and a PCV of 120 ± 05 g/L after 24 to 32 hours. Microscopic analysis reaffirmed sterility [17].

2.6. Estimating Biomass

Packed Cell Volume (PCV) was used to demonstrate how much biomass was piling up during the fermentation process.

Packed Cell Volume (PCV) Determination: To find out the Packed Cell Volume (PCV), we took a clean, dry 15 mL centrifuge tube and weighed it empty, which we called **W1.** A 10-gram sample of fermentation broth was put into the tube. For 20 minutes, the sample was spun at 5000 rpm. After the centrifugation, the supernatant was carefully poured off, and the weight of the tube with the cell pellet was noted as **W2.**

The weight of the packed cells (pellet) was calculated as:

W cells = W2−W1

Packed Cell Volume (PCV) was then expressed using the formula:

PCV (g/l) = $\frac{\mathbf{W}\ \boldsymbol{cells}}{\mathbf{W}\ \boldsymbol{Sample}}$ **x 1000**

For example: weight of an empty clean dry centrifuge tube 7.50 g (W1), weight of the sample 10 g, after centrifugation weight of the cell pellet is 9.20 g (W2)

W cells = W2−W1= 9.20 g – 7.50 g= 1.70 g

PCV (g/l) = $\frac{1.70\ \text{g}}{10\ \text{g}}$ x 1000= 0.170 g x 1000= 170 g/l

3. RESULTS

3.1. Shake Flask Level Production of Polymyxin B Sulphate:

Shake Flask-Level Production of Polymyxin B Sulphate Shake flask tests were done to see how much Bacillus polymyxa ATCC 10401 could make under the best growth conditions and with the right medium composition. The fermentation happened over 72 hours in 250 mL shake flasks, each with 25 mL of production media. The cultures were held at 30°C and stirred at 250 rpm. Over the course of 72 hours of fermentation, the amount of polymyxin B sulfate progressively rose until it reached a peak of 1.20 g/L. The culture pH slowly declined from 6.60 at the start of the batch to 5.50 at the end. This showed that the cells were breaking down and the media was getting more acidic. The packed cell volume (PCV) followed a usual pattern of microbial development. It went up during the active growth phase, peaked at 39%, and then went down a little to 25% during the stationary phase. As the cells expanded, the general patterns in pH, PCV, and product titre suggested that secondary metabolites were being generated (Figure 01). At 72 hours, the growth phase transitioned from late exponential to early stationary, and the most polymyxin B sulfate (1.20 g/L) was generated. Table 01 gives a summary of the fermentation parameters that were measured throughout time during shake flask studies. These results confirm the potential of the current culture and media conditions for further scale-up improvement.

Figure 01 shows the separate graphs for **pH, PCV,** and **Polymyxin B titre** across the 72-hour shake flask fermentation:

- The **pH** level slowly dropped from 6.60 to 5.50.
- During the growth phase, **PCV** rose steadily, reaching a high of 39% before falling significantly to 25%.
- The **titre of Polymyxin B** in the batch progressively up over time, reaching 1.20 g/L after 72 hours.
- This combined graph shows all the important fermentation parameters throughout the course of 72 hours: **pH, PCV, and Polymyxin B titre** of shake flask batch. It shows how biomass growth, medium acidification, and product creation are related to each other.

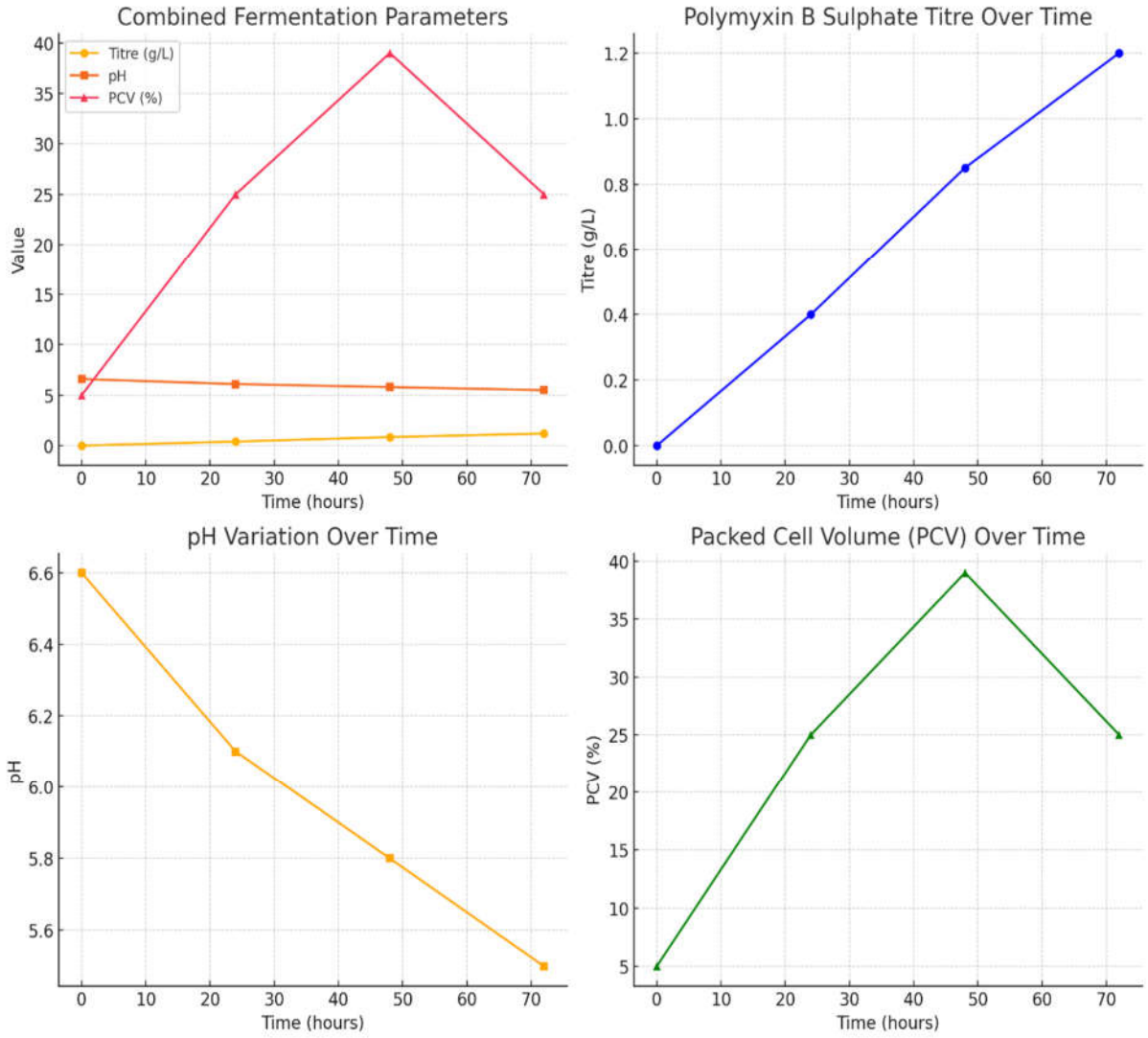

Figure 01:Tread graph of pH, PCV & Polymyxin B titre

Table 1: Shake Flask Fermentation Parameters Over Time

Time (hrs)	Titre (g/L)	pH	PCV (%)
0	0.00	6.60	5
24	0.40	6.10	25
48	0.85	5.80	39
72	1.20	5.50	25

3.2. Making Polymyxin B Sulphate on a Large Scale

After achieving good results in the shaking flask, the method was tried out with higher amounts to see how it performed. The seed culture that was cultivated in 5 L glass flasks was grown even more in a 20 L semi-automatic fermenter. After that, 10% (v/v) of it was put into a 100 L stainless steel fermentation tank for manufacturing.

The batch fermentation happened at 30°C for three days. Aeration was gradually increased from 0.70 to 1.20 VVM, and agitation was gradually modified (tip speed 1.0 to 4.0 m/s) based on the amount of dissolved oxygen (DO), notably when DO levels dropped to 30%.

The pH started off between 6.50 and 6.60, but it decreased to about 6.00 in the first 18 hours and stayed between 5.70 and 5.80 for the rest of the fermentation duration. The packed cell volume (PCV) was 198 g/L at initially. It slowly dropped down and stayed between 130 and 140 g/L at the end (Figure 02). HPLC examination showed that the level of polymyxin B sulfate steadily increased, reaching its highest point of 1.20 g/L after 72 hours (Table 02).

The scale-up process generally preserved the same physiological and production trends as those seen at the shaking flask level. This illustrates that the current setup can be used to make the process even better.

Table 2: Fermenter-Scale Batch Parameters

Time (h)	pH	PCV (g/L)	Titre (g/L)
0	6.55	198	0.05
18	6.00	170	0.30
36	5.80	150	0.65
54	5.75	140	1.00
72	5.75	135	1.20

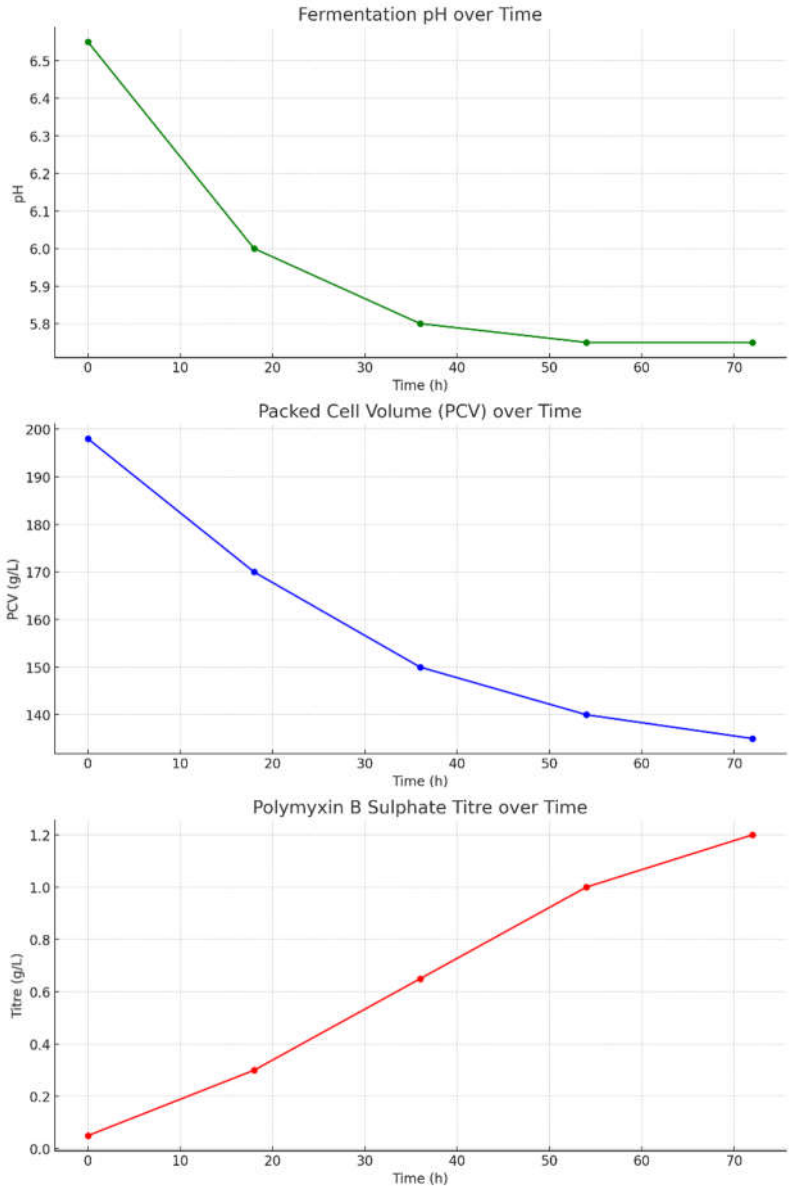

Figure 02: Fermentation Profile of Polymyxin B Sulphate Production

4. Conclusion

This paper outlines an effective mechanism for enhancing the production of polymyxin B sulphate through strategic medium modification and scalable fermentation techniques employing Bacillus polymyxa. Initial shake flask experiments utilizing a cost-effective wheat flour-based media, pretreated with α-amylase, attained titres of up to 1.20 g/L, demonstrating the effectiveness of agro-industrial substrates in promoting secondary metabolite production [10,11,16].

The culture was robust, sterile, and alive at every level of the step-by-step process for making inoculum from a lab-scale culture to a seed fermenter and finally to the production vessel. The fermentation method was effectively adapted to a 100 L semi-automated stainless-steel fermenter, preserving comparable physiological and metabolic properties throughout [12,14,17]. The last fermentation cycle

always made 1.20 g/L of polymyxin B sulfate after 72 hours. This was because the aeration, pH, and agitation parameters were just right.

Downstream processing, which includes getting rid of cells and cleaning the material, got back roughly 70% of the powder, making it safe for use in medication. This method for making polymyxin B sulfate on a large scale uses cheap resources, reliable inoculum preparation, and strict fermentation control. It could potentially be used to create additional valuable microbial bioproducts [18,19].

5. Abbreviation:

Abbreviation	Full Form
B. polymyxa	*Bacillus polymyxa*
NRPS	Non-ribosomal peptide synthetases
PCV	Packed Cell Volume
Titre	Concentration of Polymyxin B (g/L)
pH	Potential of Hydrogen
rpm	Revolutions per Minute
°C	Degrees Celsius
v/v	Volume by Volume
g/L	Grams per Liter
mL	Millilitre
HPLC	High-Performance Liquid Chromatography
DO	Dissolved Oxygen
µg/mL	Micrograms per Millilitre
m/s	Meter per second
ATCC	American Type Culture Collection

6. References

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