ORIGINAL ARTICLE

Production, separation, and antimicrobial activity assessment of pristinamycin produced using date fruit extract as substrate

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Date fruits extract was assessed as substrate for pristinamycin production in shake flasks using *Streptomyces pristinaespiralis* DSMZ 40338 as production organism. A process of microfiltration, concentration, and solvent extraction was used to recover the antibiotic from the fermentation broth and its antimicrobial activity was tested using a well assay method. During a fermentation period of 120 h, the bacterium consumed approximately 28.4 g/l sugars, grew from 0.4 g/l to 4.0 g/l dry weight, and produced 51.0 mg/L pristinamycin. The microfiltration, concentration, and ethyl acetate solvent extraction applied resulted in 71% pristinamycin recovery. The antibiotic showed inhibition capacity comparable to that of the pristinamycins IA and IIA standards. The inhibited bacteria were *Staphylococcus aureus, Escherichia coli, and Enterococcus faecium*. Clear inhibition zones of 13.3–19.6 mm diameter were formed.

Key words: Antibiotics, date fruits, fermentation, inhibition, pristinamycin, recovery

INTRODUCTION

Pristinamycin is industrially produced by microbial fermentation using the bacterium *Streptomyces pristinaespiralis*. Other *Streptomyces* strains showing promising antibiotic yields were described (Shrestha *et al.*).[1] In most cases, this antibiotic is made of two components, namely pristinamycin IA (approximately 30%) and pristinamycin IIA (approximately 70%).[2,3] The antibiotic inhibits protein synthesis in bacteria when it binds to their ribosomes.[4] Interestingly, the bacterium *Streptococcus pneumonia*, which possesses high resistance to a variety of antibiotics, is especially sensitive to this antibiotic a fact that makes it a good candidate for the control of diseases caused by this bacterium.[5-7] Monosaccharides such as glucose are the main substrate used for commercial antibiotic production. Raw materials predominantly used as sources of these sugars are molasses or hydrolyzed starches. The main concerns in the industrial production of antibiotics are the efficiency of the production organism and the optimization of the production process.[8] A potential raw material for production is date fruits, which contain 60%–80% w/w sugars, as well as a wide spectrum of minerals and B-complex vitamins. In this study, date fruits as a source of sugars, mainly glucose and fructose, were used as a substrate to produce pristinamycin.

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Submitted: 02-Feb-2022 **Revised:** 09-Mar-2022
Accepted: 16-Mar-2022 **Published:** 05-Jul-2022

Access this article online	
Quick Response Code:	Website: www.japtr.org
	DOI: 10.4103/japtr.japtr_40_22

How to cite this article: Hamad SH, Aleid SM, Elgorashe RE, Babker MY. Production, separation, and antimicrobial activity assessment of pristinamycin produced using date fruit extract as substrate. J Adv Pharm Technol Res 2022;13:161-5.

MATERIALS AND METHODS

Date syrup extraction

The syrup was extracted from "Khalas" cultivar date fruits collected by the main author from the farmer's local market in Hofuf City, Saudi Arabia, in mid-August 2019 harvest season. The process was: to each kg depicted date fruits 3 l water added, then heating at 80°C for 30 min and filter-pressing to about 20% sugar concentration with further concentration as needed.[9] Date syrup (DS) was filter sterilized to avoid the formation of materials toxic to the production organism resulting from heat sterilization. Filter sterilization done through 0.45 µm pore size filter paper (FILTROX AG, Switzerland) using a Nalgene® Rapid-Flow™ Sterile filtration unit (Thermo Fisher Scientific, USA), connected with a Thermo Scientific RV8 VLP200 Vacuum Pump. Sugars concentration was measured using high-performance liquid chromatography (HPLC) as described by Aleid.[9]

Mineral medium and MPS2 medium

The mineral medium used was developed by Mehmood *et al*.[10] It is composed of (g/l): 7.5 malonic acid, 2.0 L-arginine, 1.5 L-glutamate, 0.3 $MgSO_4.7H_2O$, 0.015 $FeSO_4.7H_2O$, 0.55 $ZnSO_4.7H_2O$, 20 3-morpholinopropanesulfonic acid, 0.5 K_2HPO_4, and 0.6 $CaCl_2.2H_2O$. The carbon and energy source was glucose, added to the mineral medium at 30 g/L, giving the medium termed MPS2. The pH of the medium was adjusted at 6.8 before sterilization for 20 min at 121°C. Each of $CaCl_2$, K_2HPO_4, and $FeSO_4 \cdot 7H_2O$ were autoclaved separately then added to the medium.

The date syrup + MPS2 medium

From HPLC analysis, the sugar content of DS was 34.7, 31.9, and 0.2 g/L glucose, fructose, and sucrose, respectively. DS added to the mineral medium as carbon and energy source formed the medium named DS + MPS2. The sugar content of this medium was approximately 30 g/L glucose and fructose each.

Pristinamycins production

The bacterium used for production was *S. pristinaespiralis* (DSMZ 40338, Germany). Each fermentation run carried out in 40 ml DS + MPS2 medium in flasks inoculated with 4 ml bacterial suspension (approximately 4.0×10^8 cells/ml), 28°C incubation temperature under shaking (250 rpm, 120 h). Samples (three replicates) drawn at 24 h intervals to measure biomass, antibiotic, and sugar concentrations.

Measurement of the concentrations of bacterial biomass and sugars

Biomass concentration measured by separating the cells by centrifugation and drying in an oven at 70°C under vacuum for 24 h. Sugar concentration determined using the Blakeney and Mutton colorimetric method.[11]

Pristinamycin harvest

Harvest performed according to Okudoh and Wallis[12] with some modifications using the solvents petroleum ether, acetone, and ethyl acetate, at 1:1 v/v solvent: Broth [Figure 1].

Pristinamycin quantitation and antimicrobial activity determination

Pristinamycin concentration was determined by HPLC according to Thibaut *et al*.[13] with some modifications as described in Aleid *et al*.[14] Pristinamycin IA and IIA (AlsaChim (Illkirch. France) used as standards. The retention times were 15.9 min for PIA and 26.7 min for PIIA. The antimicrobial activity was tested using the well assay method according to Nalawade *et al*.[15] Nutrient agar (NA, Oxoid, CM003) test plates were inoculated with freshly grown bacteria, 100 µl filtered broth applied to the wells, incubated at 30ºC for 24 h, then the inhibition zones were measured. Pristinamycins IA and IIA (50 mg/L) applied to the wells as test references in the same way. The bacteria tested were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *and Enterococcus faecium* ATCC 27270.

RESULTS AND DISCUSSION

Antibiotic production using date syrup + MPS2 medium

The growth of the bacterium, antibiotic production, and sugar consumption are shown in Figures 2-5. Data presented are averages of three replicates. The bacterium grew at a relatively low rate in the first 24 h of fermentation, then the growth rate increased during the second 24 h, and the concentration of the bacterial biomass reached approximately 2 g/l [Figure 2]. During the following 3 days of propagation, the bacterium continued to grow but at a relatively low rate and the concentration of the

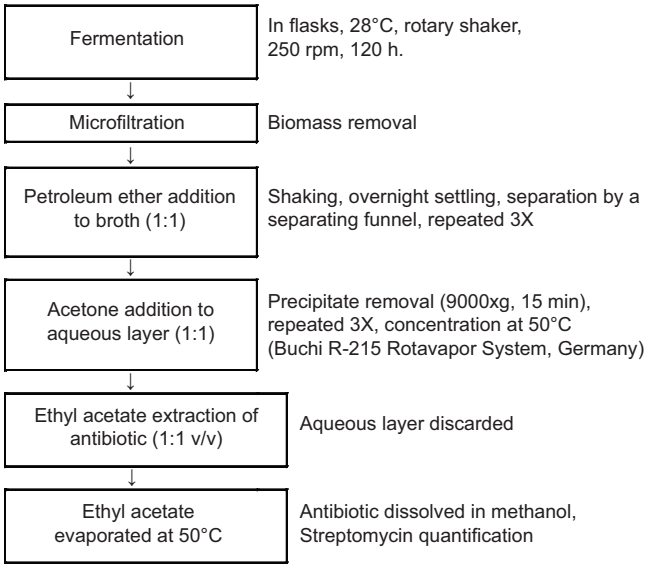

Figure 1: Extraction and recovery of the streptomycin from the fermentation broth

biomass reached approximately 4 g/l at the end of the 120-h propagation.

Concentration of pristinamycin in the fermentation broth was determined by filtration of the broth to remove the bacterial cells, then direct injection in the HPLC system. Both pristinamycin P1A and P11A were produced from MPS2 media [Figure 3]. The antibiotic production started after about 70 h of fermentation [Figure 4]. Production continued and reached a total maximum of approximately 51 mg/l consisting of 35 mg/l P1A and 16 g/l P11A after 96 h. Thereafter, no increase in antibiotic concentration was observed until the termination of the runs after 120 h. Pristinamycin concentrations of 45 up to 1130 mg/l were reported in the literature cited.[5,11,16] It is reported that *S. pristinaespiralis* produces enzymes that degrade pristinamycin during its growth and that end-product inhibition of the antibiotic production occurs resulting in the highest attainable concentrations of approximately 100 mg/l.[5]

Sugar consumption and pristinamycin yield

Sugar concentration at the beginning of fermentation was approximately 45 g/l, which decreased to 16 g/l by the end of the run [Figure 5]. Sugar consumption was high as the bacterium grew actively during the first 72, then consumption started to slow down and the final concentrations of glucose and fructose in the fermentation broth decreased to 8.8 and 7.8 g/L, respectively, after 120 h of propagation [Figure 5]. Hence, the bacterium consumed approximately 29 g/l sugar and produced 51 mg/l antibiotics from this amount of sugar consumed. This amount represents a yield of approximately 2 mg antibiotic per g sugar provided to the bacterium (2 mg/g). Theoretically, 7.5 g glucose should yield 1.0 g pristinamycin (133 mg/g) according to their molecular weights (1349.5 g pristinamycin and 180 g glucose). Hence, the actual antibiotic yield reached in this study represents only about 2% of the theory, which means that <1 g/l sugar was consumed for antibiotic production and that sugar was mainly consumed for bacterial growth and probably for the formation of metabolites other than the antibiotic. The final concentration of bacterial biomass was 4.0 g/l broth from the consumption of approximately 8 g/l sugar taking the theoretical yield of microbial biomass on sugar to be 0.5 g/g. It is assumed that the rest balance of about 20 g/l sugar has been used for the formation of bacterial biomass and probably other metabolites during the 5 days of propagation; but these cells have died and their biomass decomposed. Findings of Manteca *et al*.[17] for the bacterium *Streptomyces coelicolor*

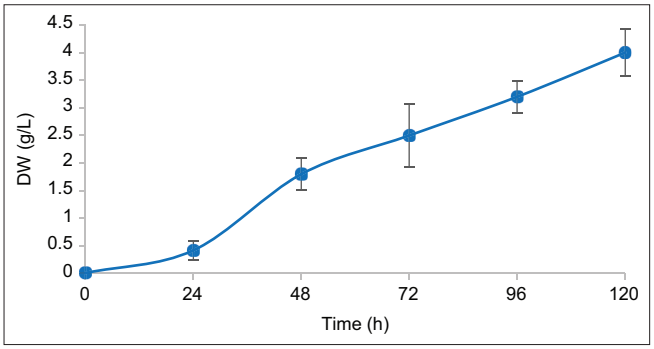

Figure 2: *Streptomyces pristinaespiralis* cell mass during the fermentation process

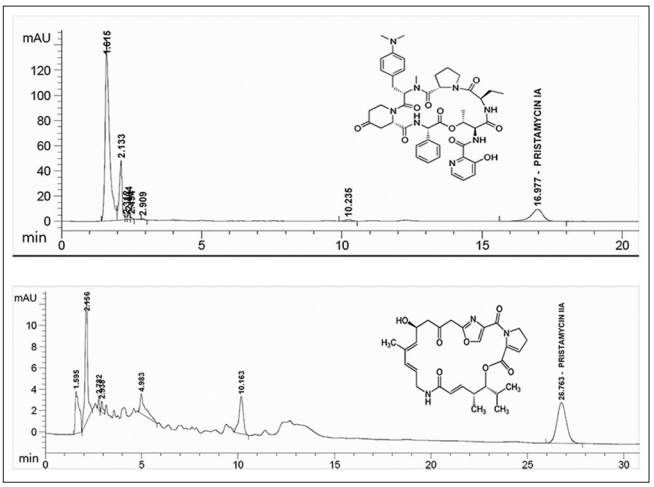

Figure 3: High-performance liquid chromatography graph shows a peak shape for pristinamycins (IA and IIA)

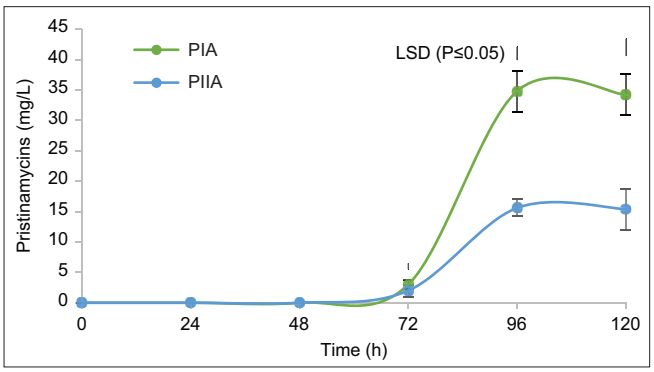

Figure 4: Production of pristinamycins during the fermentation process

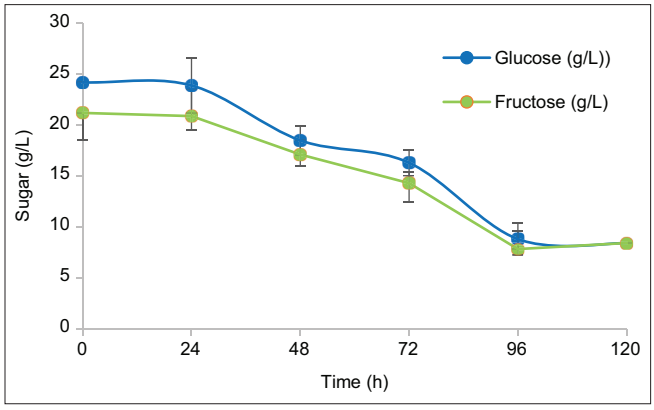

Figure 5: Sugar consumption during the fermentation process

support this assumption. These authors propagated the bacterium in submerged culture and found that it grows in cycles of pellet formation, cell death in the pellet center flowed by another growth phase.

Efficiency of pristinamycin separation and recovery

The efficiency of the process applied for the separation and recovery of pristinamycin was tested. The antibiotic was extracted from the fermented broth as described above and the dried antibiotic was dissolved in 10 ml methanol. The antibiotic content of this 10 ml methanol solution was extracted from 1.5-l broth containing 51 mg/l; hence, the solution should contain approximately 7.5 g/l pristinamycin. In parallel, 10 g/l pristinamycin was dissolved in the broth used for antibiotic production and extracted in the same way.

The content of the methanol solution determined in the HPLC was 5.3 g/l and that of the pristinamycin solution was 7.43 g/l. This means that the efficiency of the extraction method applied was about 70.7% for the fermentation broth and 74.3% for the pristinamycin solution. The two results are comparable; the lower value for the extraction from the broth could be due to the very low concentration of the antibiotic in the fermentation broth (51 mg/l). According to Okudoh and Wallis[12] separation of compounds of different polarities is enhanced by solvent extraction. The authors extracted hydrophilic organic substances from the broth with polar solvents, while the hydrophobic ones were extracted in the nonpolar solvents used. Petroleum ether, a nonpolar solvent, was used to remove lipids from the broth,

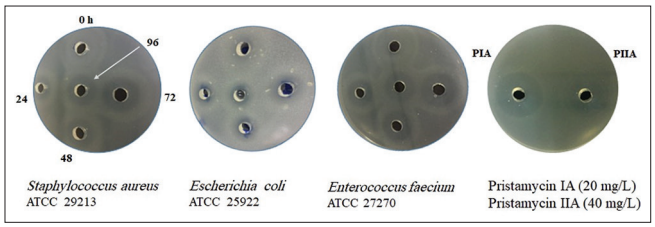

Figure 6: Inhibition zones spectrum of pristinamycins produced in date syrup +MPS2 Medium

the polar acetone to precipitate and remove proteins, and the polar ethyl acetate to extract the remaining polar organic substances. The streptomycins, including pristinamycin, are polar organic compounds soluble in polar organic solvents such as ethyl acetate and insoluble in the nonpolar solvents such as petroleum ether.[18]

Antimicrobial activity of produced pristinamycin

The antimicrobial activity of pristinamycin was assessed using the well assay method. No inhibition was seen from the broth samples taken at 0, 24, 48, and 72 h [Table 1]. The samples taken at 0, 24, and 48 h contained no antibiotic and the 72 h sample contained only traces as can be seen in Figure 4. Inhibition zones appeared from the samples taken at 96 and 120 h, where the antibiotic concentrations were approximately 51 and 50 mg/l, respectively [Table 1 and Figure 4]. The antibiotic produced in this work showed inhibition capacity comparable to that of the pristinamycins IA and IIA used as standards as could be seen from [Figures 5 and 6]. The inhibited bacteria were *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *and E. faecium* ATCC 27270.

CONCLUSION

Date fruit extract was successfully used as a source of sugar to substitute glucose in the substrate for pristinamycin produced by *S. pristinaespiralis*. The fermentation process for the antibiotic production was optimized, the antibiotic efficiently harvested from the fermentation broth and its antimicrobial activity tested. The produced antibiotic was found to inhibit the growth of the bacteria *E. coli and E. faecium*. More work is needed on the scale-up production of this antibiotic.

Acknowledgments

The authors would like to thank the Date Palm Research Center of Excellence, King Faisal University, Saudi Arabia, for the financial support, and the University of Lorraine-LRGP, France, for the technical and scientific advice.

Table 1: Diameters of inhibition zones against selected microorganisms in a well assay test

Broth taken at (h) and reference antibiotic	pH	Inhibition zone (mm)		
		Staphylococcus aureus ATCC 29213	*Escherichia coli* ATCC 25922	*Enterococcus faecium* ATCC 27270
0	4.56±0.08	nd	nd	nd
24	4.20±0.09	nd	nd	nd
48	3.33±0.12	nd	nd	nd
72	3.26±0.13	nd	nd	nd
96	3.24±0.09	18±0.90	13.3±0.80	19.6±1.00
120	3.25±0.11	17.6±1.00	13.3±1.2	18.3±1.50
Pristinamycin IA	-	23.5±1.00	14.5±1.30	26.5±0.70
Pristinamycin IIA	-	21±0.80	14.5±1.30	23.5±0.90

nd: Inhibition zone <10 mm, ATCC: American Type Culture Collection

Financial support and sponsorship

A grant from Date Palm Research Center of Excellence, King Faisal University.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Shrestha B, Nath DK, Maharjan A, Poudel A, Pradhan RN, Aryal S. Isolation and characterization of potential antibiotic-producing actinomycetes from water and soil sediments of different regions *of Nepal. Int J Microbiol 2021;2021:5586165.*
2. Corvini PF, Delaunay S, Maujean F, Rondags E, Vivier H, Ggoergen JI, *et al*. Intracellular pH of *Streptomyces pristinaespiralis* is correlated to the sequential use of carbon sources during the pristinamycins-producing process. Enzyme Microb Technol 2004;34:101-7.
3. Aumercier M, Bouhallab S, Capmau ML, Le Goffic F. Irreversible binding of pristinamycin IIA (streptogramin A) to ribosomes explains its "lasting damage" effect. J Antibiot (Tokyo) 1986;39:1322-8.
4. Ng J, Gosbell IB. Successful oral pristinamycin therapy for osteoarticular infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) and other Staphylococcus spp. J Antimicrob Chemother 2005;55:1008-12.
5. Jia B, Jin ZH, Lei YL, Mei LH, Li NH. Improved production of pristinamycin coupled with an adsorbent resin in fermentation by *Streptomyces pristinaespiralis*. Biotechnol Lett 2006;28:1811-5.
6. Reinert R. The antimicrobial resistance profile of *Streptococcus pneumoniae*. Eur Soc Clin Microbiol Infect Dis 2009;15:7-11.
7. Mehmood N, Olmos E, Goergen JL, Blanchard F, Marchal P, Klöckner W, *et al*. Decoupling of oxygen transfer and power dissipation for the study of the production of pristinamycins by *Streptomyces pristinaespiralis* in shaking flasks. Biochem Eng J 2012;68:25-33.
8. Zhang L, Zheng X, Jin Z, Hu S, He M. Optimization of fermentation conditions for pristinamycin production by immobilized *Streptomyces pristinaespiralis* using response surface methodology. Electron J Biotechnol N Am 2012;5:1-12.
9. Al Eid SM. Chromatographic separation of fructose from date syrup. Int J Food Sci Nutr 2006;57:83-96.
10. Mehmood N, Olmos E, Marchal P, Goergen JL, Delaunay S. Relation between pristinamycins production by *Streptomyces pristinaespiralis*, power dissipation and volumetric gas-liquid mass transfer coefficient, kLa. Process Biochem 2010;45:1779-86.
11. Blakeney AB, Mutton LL. A simple colorimetric method for the determination of sugars in fruit and vegetables. J Sci Food Agric 1980;31:889-97.
12. Okudoh VI, Wallis FM. Enhanced recovery and identification of a tryptamine related antibiotic produced by Intrasporangium N8 from KwaZulu-Natal, South Africa. Trop J Pharm Res 2012;11:729-37.
13. Thibaut D, Ratet N, Bisch D, Faucher D, Debussche L, Blanche F. Purification of the two enzyme system catalyzing the oxidation of the D-proline residue of pristinamycin II B during the last step of pristinamycin II A biosynthesis. J Bacteriol 1995;177:5199-205.
14. Aleid SM, Hamad SH, Delaunay S, Fick M, Olmos E. Pristinamycin production using *Streptomyces pristinaespiralis* and date sirup as substrate-process modeling, optimization, and scale-up. Prep Biochem Biotechnol 2022;1:1-7.
15. Nalawade TM, Bhat KG, Sogi S. Antimicrobial activity of endodontic medicaments and vehicles using agar well diffusion method on facultative and obligate anaerobes. Int J Clin Pediatr Dent 2016;9:335-41.
16. Voelker F, Altaba S. Nitrogen source governs the patterns of growth and pristinamycin production in '*Streptomyces pristinaespiralis*'. Microbiology (Reading) 2001;147:2447-59.
17. Manteca A, Alvarez R, Salazar N, Yagüe P, Sanchez J. Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. Appl Environ Microbiol 2008;74:3877-86.
18. Lancini G, Lorenzetti R. Biology of antibiotic-producing microorganisms. In: Lancini G, Lorenzett RI, editors. Biotechnology of Antibiotics and Other Bioactive Microbial Metabolites. New York: Plenum Press; 1993. p. 19-72.