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Systematic evaluation and optimization of crystallization conditions for vancomycin purification

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Abstract−This study describes the evaluation and optimization of a crystallizing process capable of efficiently purifying vancomycin in high purity and yield. In particular, we observed how the main process parameters influenced the formation of crystals, determined their morphology, and monitored purity and yield. Acetone was shown to be more effective than alcohol solvents for the crystallization of vancomycin. The optimal distilled water/acetone ratio, storage temperature, storage time, pH, conductivity, initial vancomycin concentration and stirrer velocity were shown to be 1 : 3.5 (v/v), 10 °C, 24 h, pH 2.5, 20 ms/cm, 0.1 g/mL, and 640 rpm, respectively. Temperature had a decisive influence on crystal formation; crystals were successfully produced at 10 °C, while at other temperatures, conglomeration, disintegration and cohesion occurred. Crystal growth developed over time and was complete at about 24 h. Vancomycin purity remained at about 97.0% irrespective of storage time while the yield increased over time, reaching a maximum of 95.0% at around 24 h, after which there was no substantial change. Crystallization occurred over a certain range of pH (2.5-3.0), but purity and yield were highest at pH 2.5. When the pH was outside this range, a conglomeration (gelation) phenomenon prevented the efficient production of crystals. Vancomycin crystals were produced irrespective of the stirrer velocity, which had no influence on purity; however, the highest yield of vancomycin was obtained at 640 rpm.

Key words: Vancomycin, Crystallization, Purification, Optimization of Process Parameters, Identification of Morphology

INTRODUCTION

Vancomycin, the first glycopeptide antibiotic to be discovered, was originally isolated from *Amycolatopsis orientalis* (syn. *Streptomyces orientalis*, *Nocardia orientalis*) by Eli Lilly in Borneo in 1956 [1,2]. Vancomycin inhibits cell wall synthesis in Gram positive bacteria, thereby causing cell death. It is widely used to treat methicillin resistant *Staphylococcus aureus* (MRSA) infection and endocarditis in patients who are allergic to penicillin and cephalosporin. In addition, vancomycin is the first therapeutic agent for MRSA infection to be widely used for preventive treatment during cardiac surgery involving an artificial implant, orthopedic surgery, and neurosurgery for the placement of a ventriculoperitoneal shunt [3].

The purification of vancomycin obtained from microbial fermentation requires several steps. For vancomycin now recorded in the United States and European pharmacopeia, the vancomycin content and the amount of total and individual impurities are strictly regulated. Using HPLC analysis suggested by the United States Pharmacopeia (USP), the vancomycin content must be greater than 88% and, among other materials that may be present, none may have a content exceeding 4%. According to the European Pharmacopeia, the vancomycin content must be greater than 93% and the presence of any other material with a content exceeding 4% is restricted in the same manner as in the USP [4]. Complying with these strict regulations necessitates several steps of isolation and purification. Generally, in producing a drug with high purity such as an antibiotic, a crystallization process is introduced as the final purification step. Crystallization is the process of precipitating and producing a compound from a liquid or gas mixture [5,6], and it corresponds to a core technology for the isolation and purification of a material as well as the control of its physical properties and morphology. This process maximizes the quality of the final product and also results in a higher value-added product. Crystallization is a simple, energy-efficient and environmentally friendly process that is widely applicable and has a low fixed investment cost [7]. Conventional approaches that introduce crystallization as the final purification process for obtaining highly pure vancomycin comprise complicated, multi-step methods that involve, for example, precipitating the crystal with phosphate after purification [8], crystallizing vancomycin as a copper salt and then substituting it with phosphate [9], or precipitating the crystal at the isoelectric point (PI) of vancomycin by adding alkali and inorganic salt at pH 5-9 to obtain a free salt, then re-crystallizing the salt with hydrochlorate or dissolving it in distilled water, reducing the pH and drying under vacuum [10]. Another disadvantage of these approaches is their low yield. During crystallization, numerous basic phenomena (nuclei production, crystal growth, aggregation, disintegration, etc.) interact in a complicated manner. It is very difficult to obtain a crystal particle having a desired characteristic (e.g., structure, size, purity) by controlling only one objective. Furthermore, these phenomena directly or indirectly depend on various external operating conditions such as the supersaturation method, the supersaturated concentration, temperature, pH, stirring, presence of impurities and additives, reactor type, and operation mode [11]. Also, studies regarding a systematic and comprehensive technology for the crystallization of vancomycin are as of yet very in-

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sufficient. In the present study, we attempted to develop a crystallization process capable of more conveniently and efficiently purifying vancomycin in high purity and yield by complementing the disadvantages of prior arts and systematically optimizing the main process parameters. To this end, we observed the influence of the main process parameters on crystal formation during the course of crystallization using a video microscope, determined crystal morphology by scanning electron microscopy (SEM) and x-ray diffractometry (XRD), and monitored purity and yield by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

1. Preparation of Vancomycin Sample

Vancomycin used in this experiment was obtained through the fermentation of the microorganism *Norcardia orientalis* isolated from soil. Bacterial cells were removed from the fermentation solution containing vancomycin, which was then purified [4]. The solution was consecutively passed through cation exchange, anion exchange and porous cation exchange resins and eluted with ammonia to obtain 88% pure vancomycin in the form of hydrochlorate. Impurities such as pigment and protein were removed using alumina and a weak acidic cation exchange resin. The resulting product was used for the crystallization process.

2. Vancomycin Analysis

An HPLC system (Hewlett Packard 1100, USA) and column (4.6 ×150 mm, 5 μm, Beckman, USA) were used for analysis of vancomycin at 280 nm using a UV detector. Two milliliters of triethylamine was mixed in 1 L of distilled water and the pH was adjusted to 3.2 with phosphoric acid to prepare an aqueous buffer. Mobile phase A was prepared by mixing 10 mL of tetrahydrofuran and 50 mL of acetonitrile in 940 mL of aqueous buffer. Mobile phase B was prepared by mixing 10 mL of tetrahydrofuran and 290 mL of acetonitrile in 700 mL of aqueous buffer. The analysis was performed in isocratic and gradient mode for 30 min according to the USP [12]. Mobile phase A was isocratic for the first 12 min and was a 100% linear gradient from 12 min to 20 min. Mobile phase B was isocratic from 20 min to 22 min. After the final 22 min, mobile phase A was isocratic for 30 min. The flow rate was 0.5 mL/min and the injection volume was 20 μL. The concentration was calculated by using the peak area acquired with the standard materials.

3. Crystallization Method

The crystallization process has an influence on numerous parameters, which, in turn, have a direct influence on the structure, size, distribution, purity and yield of crystal particles [11]. This study attempted to optimize the main process parameters of the crystallization process (type of organic solvent, solvent/distilled water ratio, storage temperature and time, pH, conductivity, initial concentration, and stirrer velocity) to obtain high-purity vancomycin. A schematic view of this process is shown in Fig. 1. The reactor size and experimental volume are 90 mL and 13.5 mL, respectively. First, the sample (88% pure vancomycin) was dissolved in distilled water wherein the pH and conductivity were adjusted with 1 N hydrochloric acid and sodium chloride, respectively. Since sodium chloride has no influence on H^+, the pH was adjusted first. To efficiently induce crystal formation by a reduction in solubility, the vancomycin solution was slowly added drop-wise in several kinds of organic solvents (acetone, methanol, ethanol or propanol) while stirring. Then, a crystallization experiment was carried out by varying the storage temperature (0, 5, 10, 15, or 25 °C) and time (6, 12, 18, 24, or 30 h). After crystallization, the parent solution including the solvent adhered to crystal surfaces; thus, it was removed. The vancomycin was then washed with the organic solvent used for the crystallization process in order to obtain a clear, final crystal product. Impurities were removed from crystal surfaces by filtration through filter paper (150 mm, Whatman) [13], then the filtrate was dried under vacuum at 35 °C for 24 h and analyzed by HPLC.

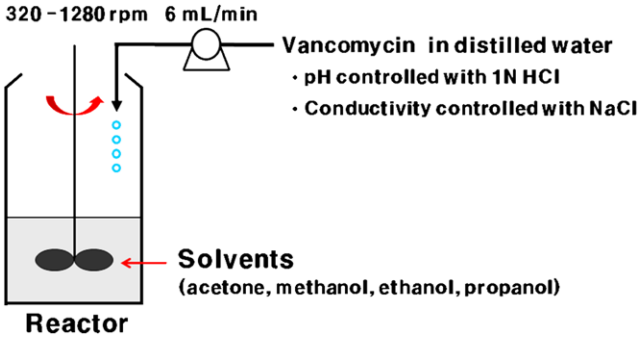

Fig. 1. Schematic diagram of crystallization process for purification of vancomycin.

To determine the morphology and size of vancomycin particles formed during crystallization, a video microscope (SV-35 Video Microscope System; Sometech., Korea) was used. Precipitates were observed at high magnification (×500). The morphology and size of the crystals were determined from video images using the IT-Plus System (Some Tech., Korea).

4. SEM and XRD Analysis

Vancomycin morphology and particle surfaces were observed at various magnifications using SEM (MIRA LMH, Tescan, Czech Republic) with accelerating voltages of 10-15 kV and an approximately 1 mg sample. Also, whether or not the structure of the vancomycin was crystalline was determined by means of an x-ray diffractometer (SMD 3000, SCINCO, Italy) operated by the WIN-HRD 3000 program at 40 kV and 40 mA with a range of 2-theta from 5 to 85°.

RESULTS AND DISCUSSION

1. Effect of Solvent Type and Distilled Water/Solvent Ratio

The degree of supersaturation during isolation/purification via solution crystallization is an important parameter that has an influence on the purity of the crystal [14,15]. Supersaturation refers to an unstable solution state in which the amount of solute present exceeds the dissolving capacity of the solvent. Reducing solubility by the addition of an organic solvent leads to supersaturation [16]. The type and amount of organic solvent in the solution crystallization are important parameters. Accordingly, solution crystallization using an appropriate water-soluble organic solvent can optimize particle size, crystal size, crystal morphology, yield and purity. Therefore, a crystallization experiment was performed with acetone or an organic alcohol solvent (methanol, ethanol, or propanol) widely used for crystallization. Concurrently, the effect of the distilled water/sol-

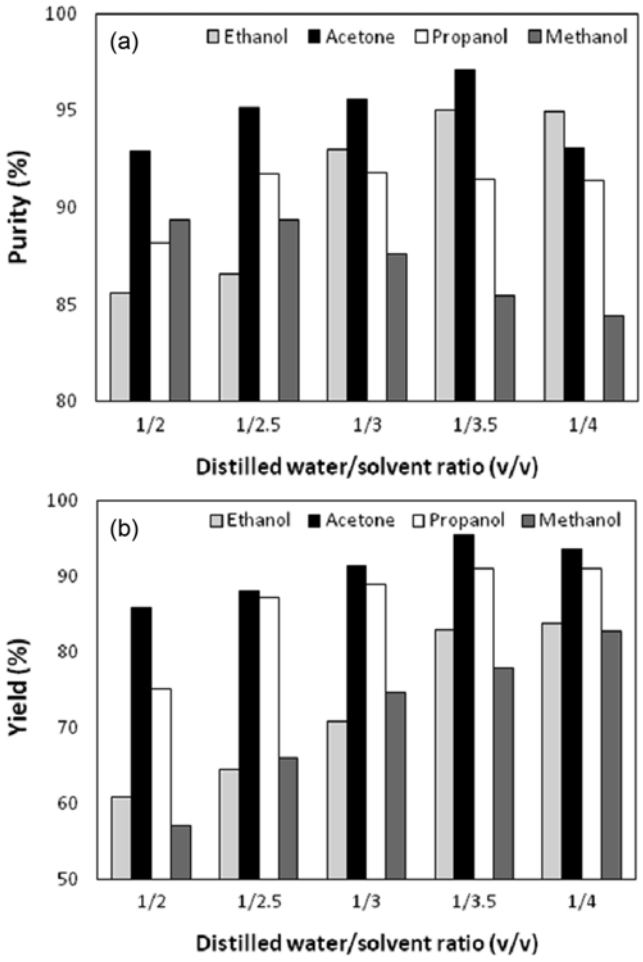

Fig. 2. Effect of distilled water/solvent ratio on purity (a) and yield (b) of vancomycin. The storage temperature, storage time, pH, conductivity, initial concentration, and stirrer velocity were 10 °C, 24 h, 2.5, 20 ms/cm, 0.1 g/mL, and 640 rpm, respectively.

vent ratio was determined using vancomycin at an initial concentration of 0.1 g/mL. Vancomycin was dissolved in distilled water, and the distilled water/solvent ratios (v/v) were 1 : 2.0, 1 : 2.5, 1 : 3.0, 1 : 3.5, 1 : 4.0. As shown in Fig. 2, a higher purity and yield were obtained by using acetone than with the organic alcohol solvents. Also, when the distilled water/acetone ratio was 1 : 3.5, the highest purity (97.1%) and yield (95.4%) were obtained. These results comply with those of prior reports [4,17] and show that, among several organic solvents, acetone was the most effective for the crystallization of vancomycin. Accordingly, the distilled water/acetone ratio was set to 1 : 3.5 and the following experiments were carried out.

2. Effect of Storage Temperature and Storage Time

For most bioproducts, solubility is sensitive to changes in temperature. In this case, temperature has a direct influence on the crystallization process and yield [11] and is thus an important parameter. To determine the variation in purity and yield of vancomycin according to changes in storage temperature, a crystallization experiment was carried out at 0, 5, 10, 15, and 25 °C. As shown in Fig. 3, storage at 10 °C resulted in the highest purity (97.8%) and yield (95.1%). After 24 h of crystallization, vancomycin crystals were observed

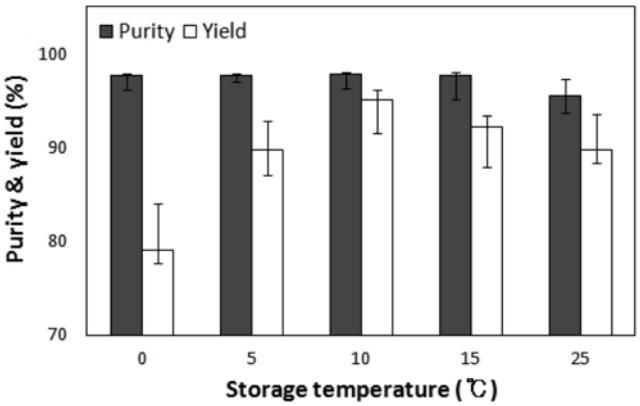

Fig. 3. Effect of storage temperature on purity and yield of vancomycin. The distilled water/acetone ratio, storage time, pH, conductivity, initial concentration, and stirrer velocity were 1 : 3.5 (v/v), 24 h, 2.5, 20 ms/cm, 0.1 g/mL, and 640 rpm, respectively.

by video microscope (Fig. 4). Crystal nuclei, the minimum units of crystal particles formed first in solution, were stably produced and grew at a storage temperature of 10 °C. Such nuclei are stable only at a certain temperature (less than the melting temperature); the presence of heat energy at a temperature exceeding the above is problematic [18]. At storage temperatures of 15 and 25 °C, the crystals were small and needle-like. Under these circumstances, the crystals disintegrated or the disintegrated crystals aggregated. Also, at 0 and 5 °C, gelation (a conglomeration phenomenon) occurred in which the crystal particles adhered to each other because the density of the crystals in solution (quantity/volume) increased [13]. In this manner, crystal structures can arise that differ from each other in the solid phase despite originating from the same materials, a property referred to as polymorphism. The generation of polymorphs depends on recrystallization due to solubility differences caused by structural instability. In the drug and pharmaceutical industries in particular, polymorphism has a very important meaning. Physical and chemical properties can differ greatly depending on crystal structure. A drug will have pharmaceutical effects when it has a certain crystal structure but may have toxic effects when it has another structure [18].

The results of SEM analysis of vancomycin crystals are shown in Fig. 5. SEM can capture three-dimensional and fine structures not visible by video microscopy. When the storage temperature was 10 °C, it was possible to identify a crystalline morphology. Crystalline particles of vancomycin are evident in Fig. 5(c), which was confirmed by XRD analysis (Fig. 6). In the XRD patterns, we could see meaningful peaks at 31.7° and 45.4° two theta degrees in Fig. 6(c). In the case of storage at temperatures other than 10 °C, the graph indicates the presence of all non-crystalline forms, while the peak corresponding to the crystalline form was produced by samples stored at 10 °C. According to a previous report [4], the optimal temperature for vancomycin crystallization was 2-8 °C at pH 2-5; however, as shown in Figs. 4-7, in light of the purity, yield and crystal morphology as well as the appearance of gelation phenomena at temperatures below 10 °C, there was difficulty in even recovering vancomycin through filtration after crystallization. Therefore, it was

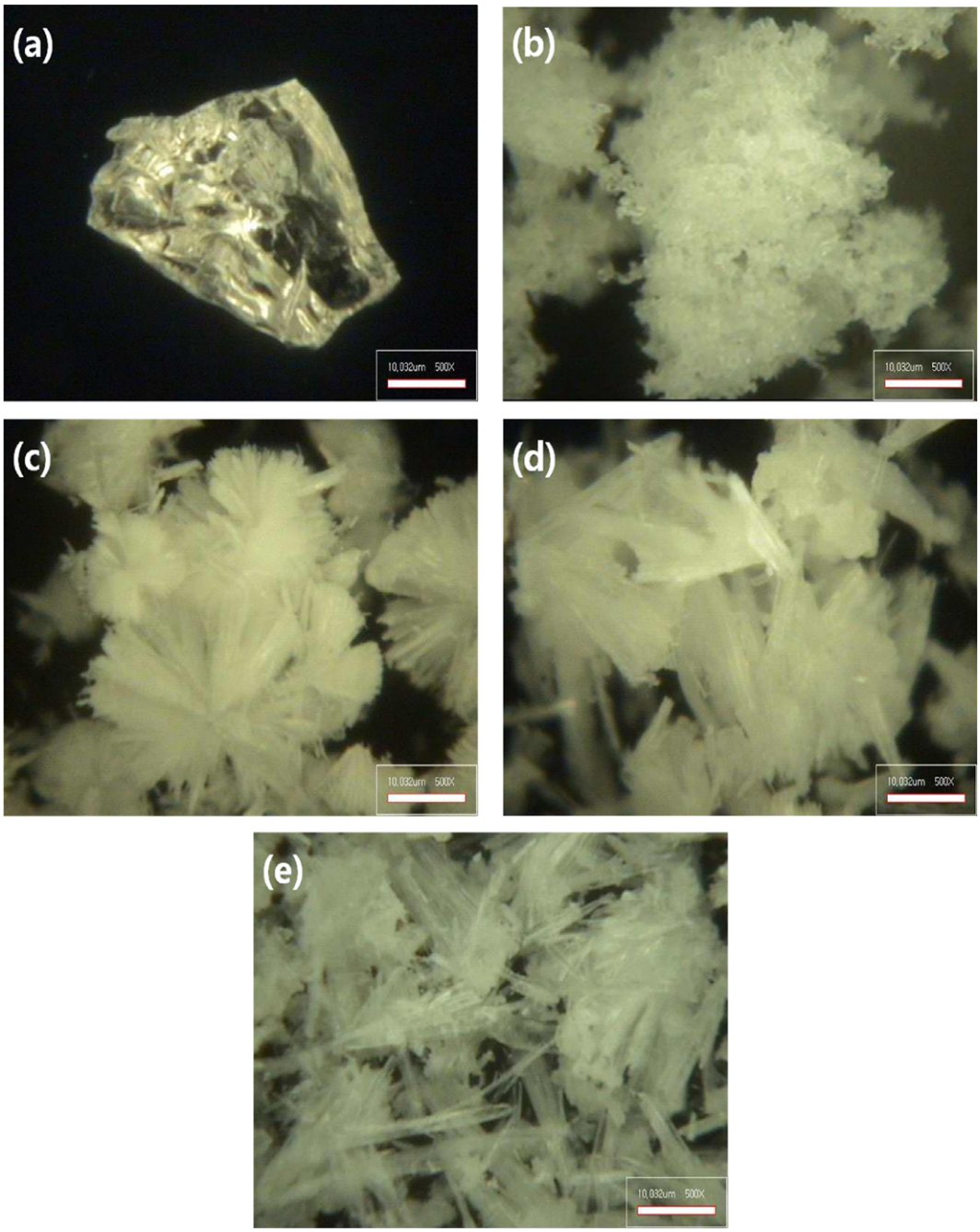

Fig. 4. Video microscope images of vancomycin crystals stored at various temperatures. The storage temperatures were 0 (a), 5 (b), 10 (c), 15 (d), and 25 °C (e).

concluded that the optimal temperature for crystallization of vancomycin is 10 °C.

To determine the effect of the crystallization time, the ratio of distilled water/acetone and crystallization temperature were set to 1 : 3.5 (v/v) and 10 °C, respectively. As shown in Fig. 7, the purity of vancomycin was about 97.0% at all crystallization times. In contrast, the yield gradually increased over time, reaching a maximal value of 95.0% after 24 h of crystallization, after which there was almost no change. Also, as shown in Fig. 8, it was determined that vancomycin crystal growth developed over time; nuclei appeared after 18 h of crystallization and gradually grew. Crystals formed after storage for more than 24 h. These results are similar to those of a report [4] in which crystallization required more than 20 h. Accordingly, the optimal storage time was determined to be 24 h.

3. Effect of pH and Conductivity

pH is another important parameter in the crystallization process, having a direct influence on the yield of bioproduct. Generally, for a bioproduct near the isoelectric point (pI), electrostatic power is minimized and solubility is maximized. Accordingly, to obtain the maximal yield, it is advantageous to induce crystallization at a pH wherein the solubility of the solution is minimized [11]. At acidic pH, vancomycin is considered to be poorly soluble because of its

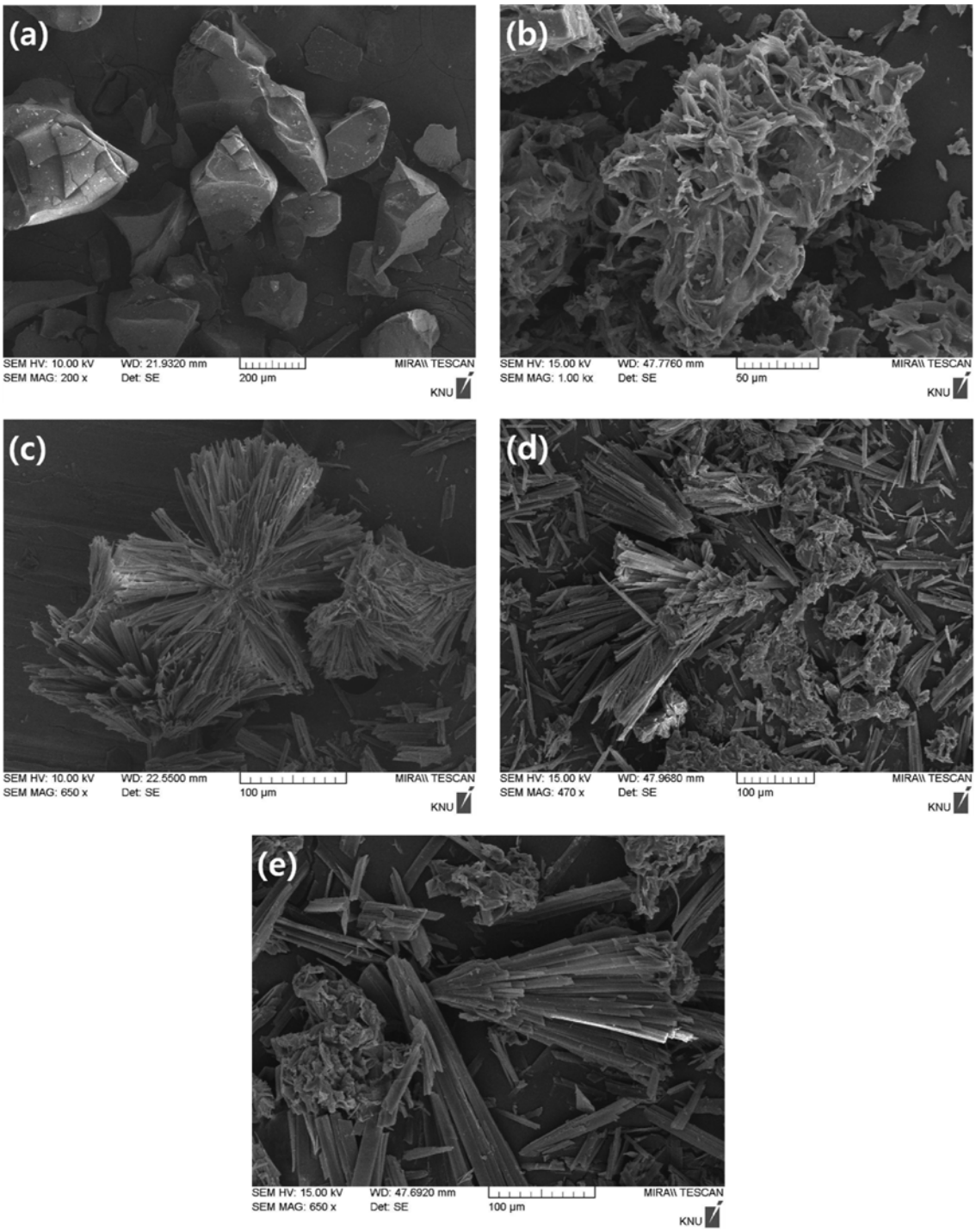

Fig. 5. SEM images of vancomycin crystals stored at various temperatures. The storage temperatures were 0 (a), 5 (b), 10 (c), 15 (d), and 25 °C (e).

pI value (8.1). Also, vancomycin is known to be stable at pH 2-7 [19]. Therefore, the pH of the distilled water was adjusted to 2.0, 2.5, 3.0 or 3.5 and crystallization was carried out. As shown in Fig. 9, when the pH was 2.5, the highest purity (97.0%) and yield (95.1%) were obtained. Also, as shown in Fig. 10, vancomycin crystals were produced at pH 2.5 and 3.0 while conglomeration occurred at other pHs. According to the literature, the optimal pH for crystallization of vancomycin is 2.0-5.0; the results of the present study fall in this range. Accordingly, pH 2.5 was selected as the optimal condition.

The effect of conductivity (10, 15, 20, or 25 ms/cm) on the crystallization of vancomycin and the purity and yield thereof was also determined. As shown in Fig. 11, when the conductivity was 20 ms/cm, the highest purity (97.0%) and yield (95.1%) were obtained, indicating that this level was the most effective for the crystallization of vancomycin. In addition, vancomycin crystals were produced throughout the range of conductivity values (data not shown).

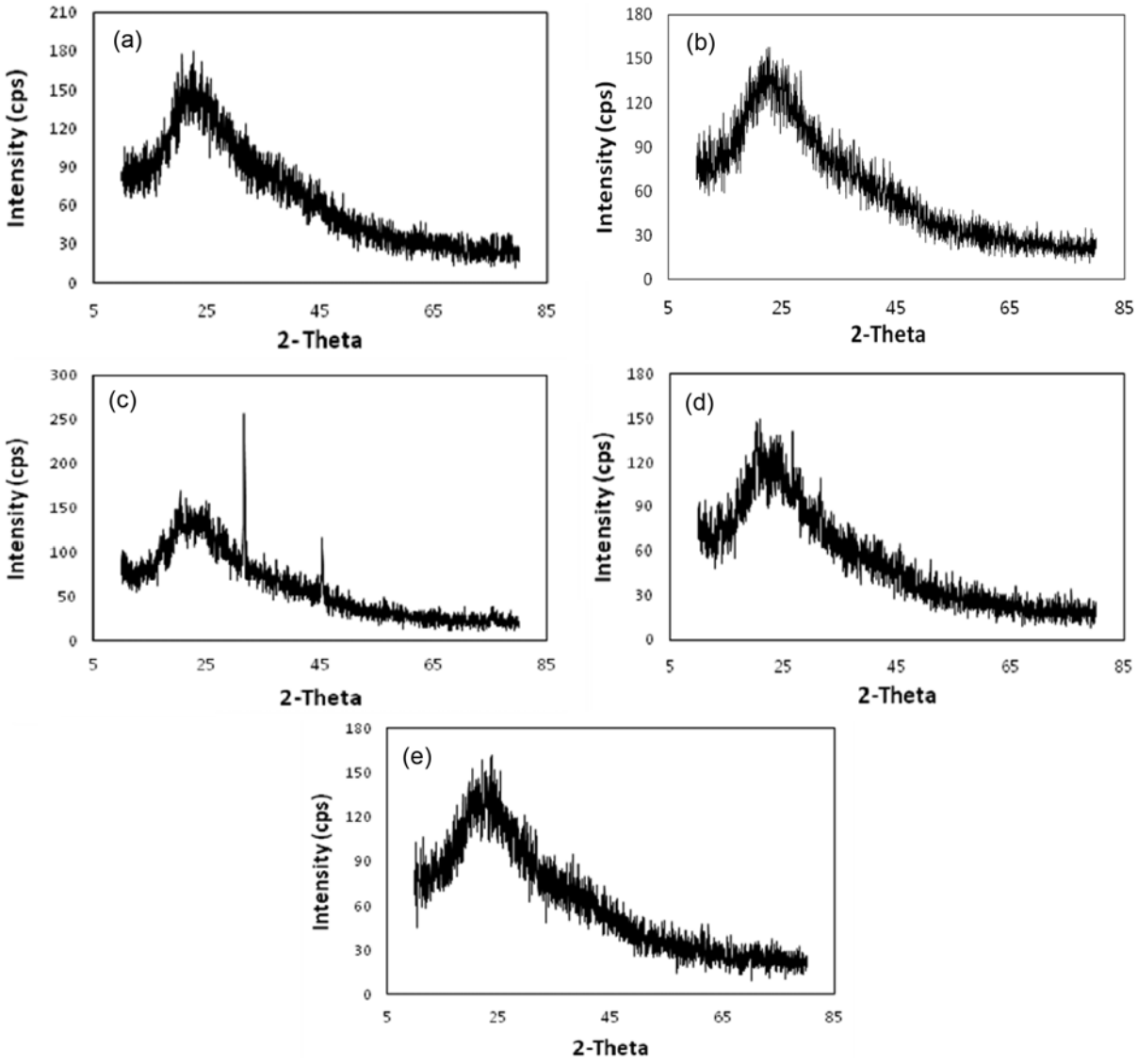

Fig. 6. XRD patterns for vancomycin crystals stored at various temperatures. The storage temperatures were 0 (a), 5 (b), 10 (c), 15 (d), and 25 °C (e).

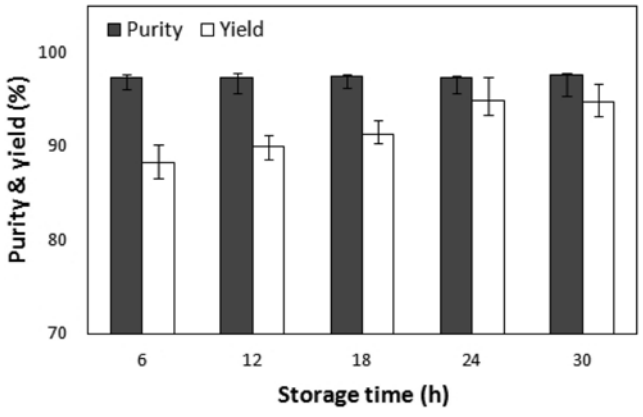

Fig. 7. Effect of storage time on purity and yield of vancomycin. The distilled water/acetone ratio, storage temperature, pH, conductivity, initial concentration, and stirrer velocity were 1 : 3.5 (v/v), 10 °C, 2.5, 20 ms/cm, 0.1 g/mL, and 640 rpm, respectively.

4. Effect of Initial Concentration and Stirrer Velocity

To determine whether the initial concentration of vancomycin has an effect on crystallization, the concentration was set to 0.05, 0.10 or 0.15 g/mL and crystallization was carried out under the above-determined optimal conditions. As shown in Fig. 12, using an initial concentration of 0.1 g/mL, the highest purity (97.6%) and yield (95.0%) of vancomycin were obtained. Also, crystals were produced using all three initial concentrations (data not shown). This result is in agreement with a previously reported range of optimal initial concentrations, 0.085 g/mL-0.160 g/mL [20].

When the stirrer velocity is changed in the crystallization process, the fluid dynamics of the solution change as a result. This has an effect firstly on the growth and aggregation of crystals and also on the production of crystal nuclei [11]. Accordingly, the effect of the stirrer velocity (320, 640, 960 or 1280 rpm) during the addition of vancomycin solution to acetone on crystallization was determined. As shown in Fig. 13, at all given stirrer velocities, the purity of vancomycin was about 97.0%. The highest yield, 95.5%, was obtained at

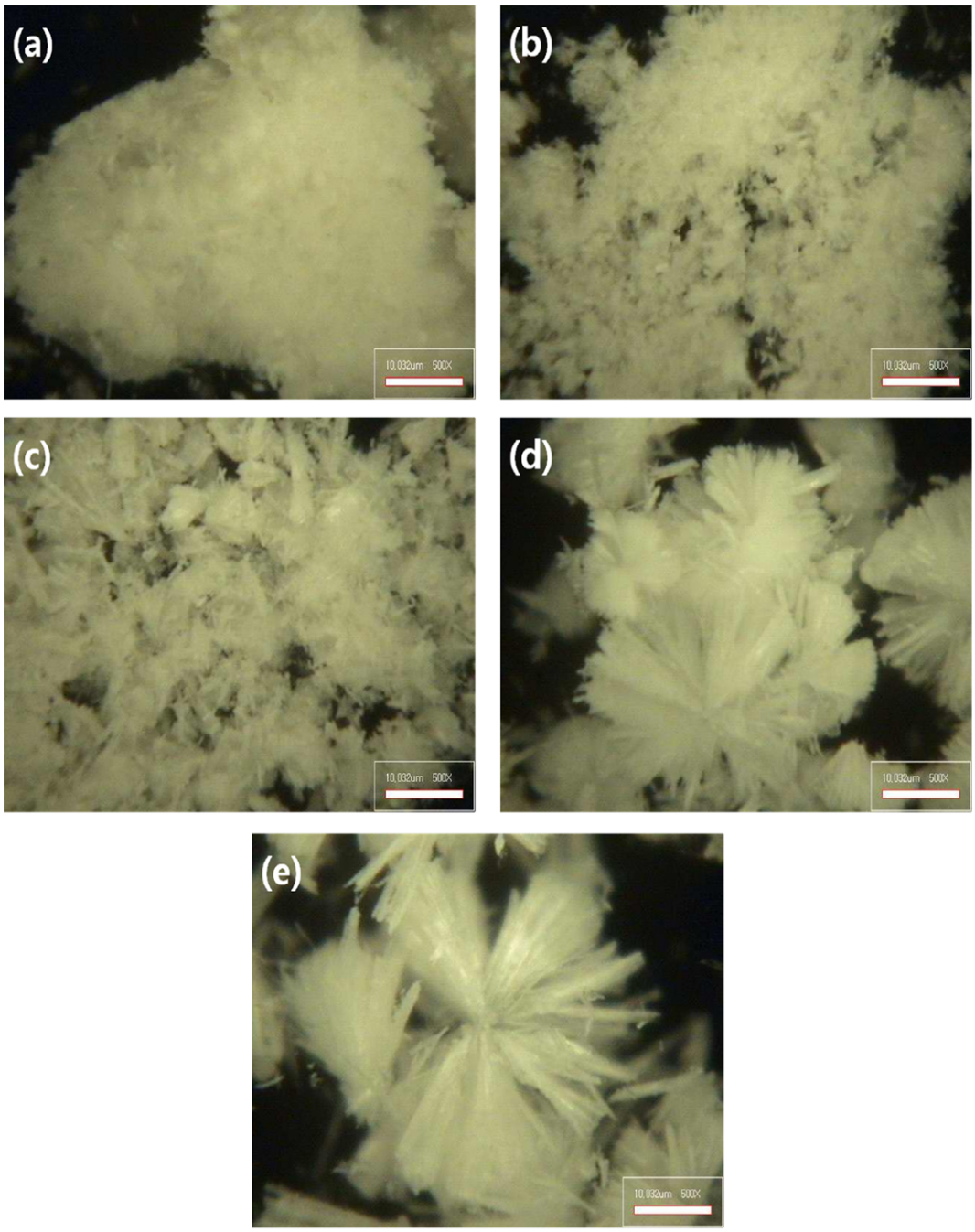

Fig. 8. Video microscope images of vancomycin crystals at various storage times. The storage times were 6 (a), 12 (b), 18 (c), 24 (d), and 30 h (e).

640 rpm. Also, vancomycin crystal nuclei were produced and grew throughout the range of stirrer velocities (data not shown).

CONCLUSIONS

We developed and optimized a crystallizing process capable of efficiently purifying vancomycin, a glycopeptide antibiotic that inhibits cell wall synthesis in Gram positive bacteria, in high purity and yield. In doing so, we have observed how the main process parameters have an influence on the formation of crystals during crystallization using video microscopy, SEM and XRD analysis.

In this study, acetone rather than an alcohol solvent was shown to be the most effective for crystallization of vancomycin, resulting in higher purity and yield. Also, the optimal distilled water/acetone ratio was determined to be 1 : 3.5 (v/v), giving the highest purity (97.1%) and yield (95.4%) of vancomycin. Crystals were produced at a storage temperature of 10 °C while at other temperatures, conglomeration, disintegration and cohesion phenomena occurred; thus, temperature had a decisive influence on vancomycin crystal formation. The highest purity (97.8%) and yield (95.1%) were obtained at 10 °C as well. Crystal growth progressed according to the storage time, and crystal production was complete at around 24 h. The

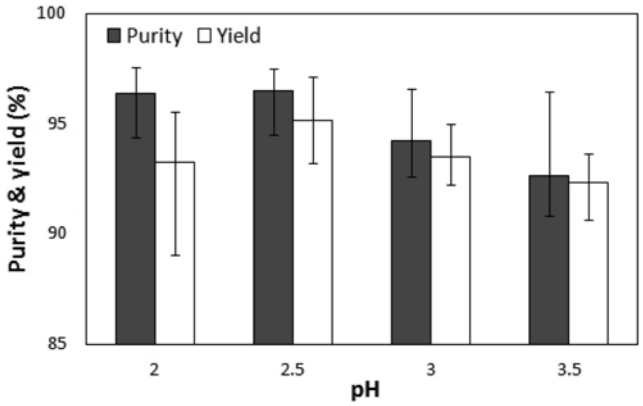

Fig. 9. Effect of pH on purity and yield of vancomycin. The distilled water/acetone ratio, storage temperature, storage time, conductivity, initial concentration, and stirrer velocity were 1 : 3.5 (v/v), 10 °C, 24 h, 20 ms/cm, 0.1 g/mL, and 640 rpm, respectively.

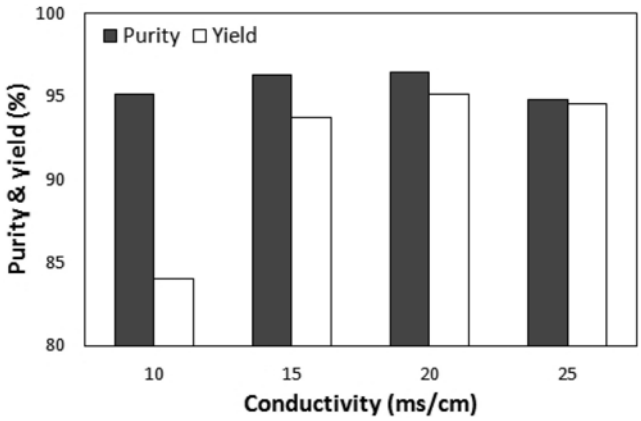

Fig. 11. Effect of conductivity on purity and yield of vancomycin. The distilled water/acetone ratio, storage temperature, storage time, pH, initial concentration, and stirrer velocity were 1 : 3.5 (v/v), 10 °C, 24 h, 2.5, 0.1 g/mL, and 640 rpm, respectively.

purity remained at about 97.0% irrespective of storage time. On the other hand, the vancomycin yield increased over time, reaching the maximal value of 95.0% around 24 h, with no substantial change occurring thereafter. Vancomycin crystallization was successful in a certain pH range (2.5-3.0), but when the pH was 2.5, the highest purity (97.0%) and yield (95.1%) of vancomycin could be obtained. When the pH was outside this range, it was difficult to produce crystals due to conglomeration. The highest purity (97.0%) and yield (95.1%) of vancomycin crystals were obtained at a conductivity of 20 ms/cm. When the initial concentration of vancomycin was 0.1 g/mL, the highest purity (97.6%) and yield (95.0%) were achieved. Vancomycin crystals were produced irrespective of the stirrer velocity.

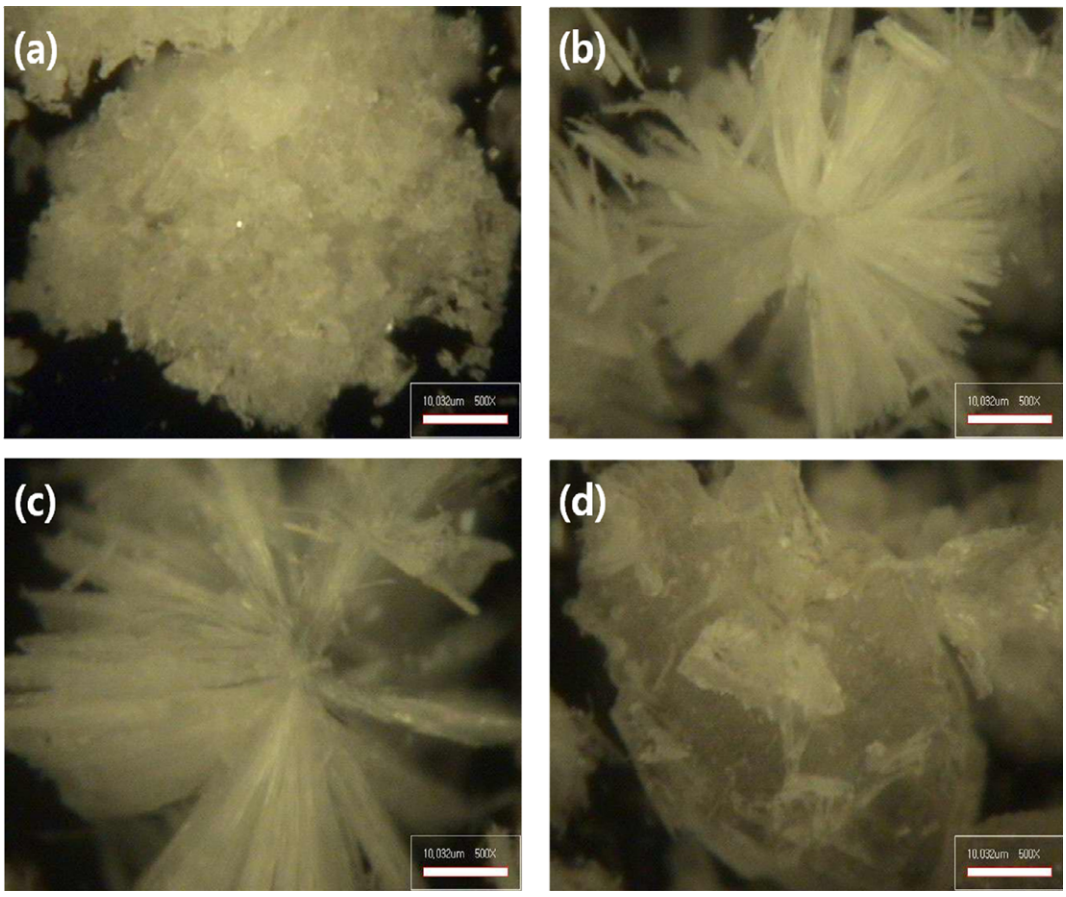

Fig. 10. Video microscope images of vancomycin crystals at various pHs. The pHs were 2.0 (a), 2.5 (b), 3.0 (c), and 3.5 (d).

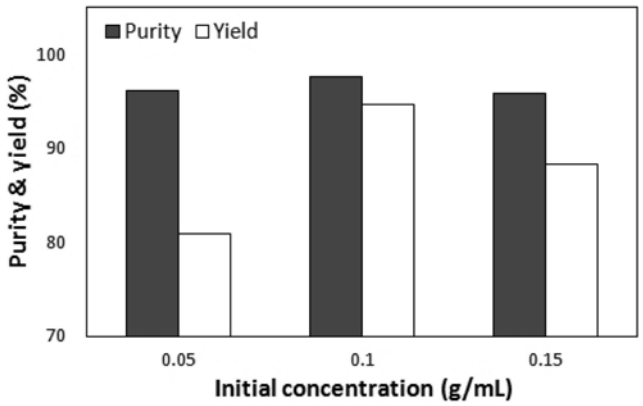

Fig. 12. Effect of initial concentration on purity and yield of vancomycin. The distilled water/acetone ratio, storage temperature, storage time, pH, conductivity, and stirrer velocity were 1 : 3.5 (v/v), 10 °C, 24 h, 2.5, 20 ms/cm, and 640 rpm, respectively.

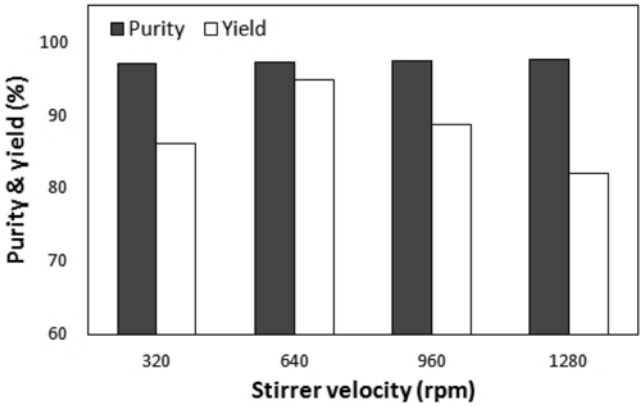

Fig. 13. Effect of stirrer velocity on purity and yield of vancomycin. The distilled water/acetone ratio, storage temperature, storage time, pH, conductivity, and initial concentration were 1 : 3.5 (v/v), 10 °C, 24 h, 2.5, 20 ms/cm, and 0.1 g/mL, respectively.

The purity (97.0%) was unaffected by the stirrer velocity, whereas the highest yield (95.0%) could be obtained at 640 rpm. Using the above optimal crystallization conditions, >97% pure vancomycin could be effectively purified with a yield of >95% from 88% pure vancomycin. Therefore, it was concluded that this method could be effectively applied to the mass production of vancomycin in the future.

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