Separation of scleroglucan and cell biomass from *Sclerotium glucanicum* grown in an inexpensive, by-product based medium

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Abstract: Scleroglucan is an extracellular polysaccharide produced by the fungi Sclerotium. Scleroglucan is stable over a broad range of temperatures, pH and salt concentration, thus having great potential in many diverse applications. Common media for scleroglucan production contain expensive components, such as yeast extract, that make the medium cost-ineffective for some industrial applications. Thus we developed a medium which uses Condensed Corn Solubles (CCS), a nutrient-rich byproduct of corn-based ethanol production, to replace expensive components. Methods typically used to recover scleroglucan are also expensive, and can limit commercialization. To evaluate alternative processes for scleroglucan recovery, we prepared scleroglucan in a modified version of Wang's medium and a CCS-glucose medium. Broth samples were initially subjected to the standard recovery method to develop a complete mass balance, and then we evaluated various treatments to improve scleroglucan recovery. These included heat treatments to lyse cells, using different alcohol precipitants, freezing or refrigerating before recovery, and diluting broth to enhance cell separation. The CCS medium produced 14.2 g/L scleroglucan, compared to 10.1 g/L in the modified Wang's medium. Based on the standard recovery protocol, we determined that 96% of the scleroglucan was recovered from the initial centrifugation and precipitation. Washing the cell pellet with water and recentrifuging only recovered a minimal amount of scleroglucan, and thus it could be eliminated from protocol to save energy and costs. Lysing cells by boiling or autoclaving did not release more scleroglucan than un-heated samples, and we also found no statistical difference between ethanol, isopopanol, and methanol as scleroglucan precipitants. Refrigerating the broth prior to scleroglucan recovery had no significant effect, while freezing actually decreased scleroglucan recovery. Initially diluting the broth by 0.50 or 0.34 resulted in the greatest scleroglucan recovery, while higher or lower dilutions decreased recovery. The optimum protocol for scleroglucan recovery was a 0.50 dilution of broth prior to centrifugation, no washing of the cell pellet, and use of the least expensive alcohol to precipitate scleroglucan from the supernatant.

Keywords: scleroglucan, *Sclerotium glucanicum*, exopolysaccharide separation, condensed corn solubles

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1 Introduction

Scleroglucan is an extracellular polysaccharide produced by fungi, including *Sclerotium, Corticium and Sclerotinia*. It comprises a β–(1,3)-glucose backbone, with single D-glucopyranosyl side groups attached with β–(1,6) bonds every third or fourth glucopyranosyl molecule on the main chain. Scleroglucan gels are stable

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over a broad range of temperatures (0–120℃), pH (1–12.2), and salt concentration (0–20%)[1-8]. Thus, scleroglucan has great potential for use in many diverse applications in petroleum recovery, agriculture, food and pharmaceutical industries[2, 9-17]. Unfortunately, its high cost has thus far limited commercial use.

The commonly cited laboratory medium for scleroglucan production was developed by Wang and McNeil [17-19] and contains yeast extract, various minerals, and glucose or sucrose. We eliminated most of the minerals and still found that a modified version of Wang's medium (containing 30 g/L glucose, 1 g/L yeast extract, and 1 g/L $MgSO_4$) was still too expensive for use on an industrial scale [20]. Based on a scleroglucan yield of 13 g/L, we estimated that the medium cost alone was $3.36/kg scleroglucan produced.

Selbmann et al.[21] evaluated a range of raw and hydrolyzed starch materials to replace glucose in Wang's medium. They found that partially or totally hydrolyzed corn starch could effectively replace glucose, but the medium still contained expensive ingredients such as yeast extract. Therefore, we evaluated a low-value byproduct of corn-based ethanol production to see if it would support growth and scleroglucan production by *Sclerotium glucanicum* without addition of expensive minerals or yeast extract[20]. This byproduct, condensed corn solubles (CCS), results when ethanol is distilled from the corn fermentation broth, solids are recovered by centrifugation and evaporators concentrate the resulting supernatant. CCS contains moderate to high levels of protein, sugars, complex carbohydrates, vitamins, various micro- and macro-nutrients, as well as lysed yeast cells[22-24]. In trials with the CCS-based medium, we achieved scleroglucan yields of 26 g/L, which reduced the medium cost to $0.37/kg scleroglucan, an almost ten-fold decrease [20].

Scleroglucan recovery from culture broth is another significant production cost. The typical method to recover high purity scleroglucan for analytical purposes involves first diluting the culture broth by 1:2 to 1:3 to reduce viscosity. Centrifugation is then used to remove the cell biomass, and the pellet is washed in an equal volume of water and recentrifuged to remove any adhering gum. The pellet can then be dried to determine dry cell weight. The cell-free supernatant fractions are then mixed with two volumes of ethanol, which causes scleroglucan to precipitate out in a gel-like form. The precipitate is recovered by vacuum filtration and can be washed once with the solvent to increase purification. The precipitate is then dried to a constant weight[2,5,10,16,18,19,25-28].

To reduce the costs for scleroglucan recovery, while still maintaining acceptable gum concentration and purity, we evaluated a range of process variables. These included broth dilution, various precipitation treatments, and thermal treatments. Our goal was to reduce the number of steps required, but also to optimize the process so that it would work with culture broth prepared from the CCS byproduct media that contains low levels of suspended solids.

2 Materials and methods

2.1 Organism, culture maintenance and media formulations

Sclerotium glucanicum NRRL 3006 was obtained from the USDA National Center for Agricultural Utilization Research (NCAUR), Peoria, IL. For long-term storage, both wild-type and Condensed Corn Solubles (CCS) acclimated strains were grown on potato dextrose agar (PDA) slants, covered with sterile mineral oil, and stored at 4℃. Cultures were routinely transferred(72-96 h) in culture media described below and incubated at 28℃ in a rotary shaker (250 r/min).

Wang's medium[17-19] was used as a control. Wang's medium contains (per L): 30.0 g glucose, 3.0 g $NaNO_3$, 1.0 g yeast extract, 1.0 g $K_2HPO_4 \cdot 3H_2O$, and 1.0 g $MgSO_4 \cdot 7H_2O$. A modified version of Wang's medium (30.0 g glucose, 1.0 g yeast extract, and 1.0 g $MgSO_4 \cdot 7H_2O$ per L) was also used for comparison. The CCS-based medium[20] contained 160 g/L pre-filtered wet weight CCS and 20 g/L glucose. CCS was obtained from a local dry-mill ethanol plant and was refrigerated until use at 4℃. After the CCS and glucose were mixed, the broth was filtered through Whatman #113 filter paper to remove the suspended solids to prevent interference with biomass quantification. All media formulations

were adjusted to pH 4.5 with either 7.0 M H_2SO_4 or 7.0 M NaOH.

2.2 Scleroglucan production conditions

To prepare large quantities of cultured broth for scleroglucan recovery trials, 3 L of media were produced in a 5 L BioFlo III reactor operated at 28℃, 350 r/min, and an aeration rate of 1 volume/volume/min (vvm) of sterile air. Trials were inoculated with a 5% inoculum of a 48 h culture grown in the corresponding medium to the trial. Cultures were incubated for 144 h, and samples were taken at 12 h intervals and analyzed as described below.

2.3 Analysis of standard scleroglucan and cell biomass recovery method

The standard method for scleroglucan and cell biomass fractionation includes diluting and centrifuging culture broth, washing the cell pellet, then recentrifuging to collect the gum-free cell pellet. Supernatant fractions are then mixed with two volumes of ethanol to precipitate scleroglucan, with the precipitate recovered by vacuum filtration. Solids are dried to a constant weight at 55℃[5,18,19,25-27]. To evaluate the effectiveness of this standard method, we followed the procedure shown in Figure 1, which includes measuring filtrate dry weights to complete the mass balance. We evaluated this method on both the modified Wang's medium and the filtered CCS medium which had been cultured as described above. Three replications were conducted for each treatment.

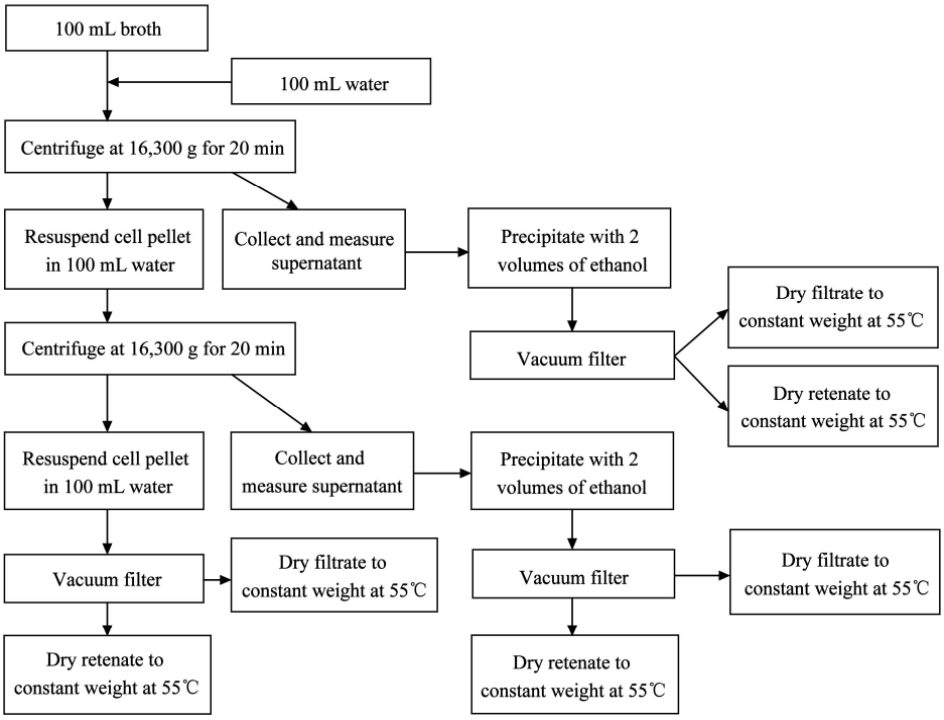

Figure 1 Standard scleroglucan and cell biomass recovery method

2.4 Effect of cell lysis and alcohol precipitant on scleroglucan recovery

Due to a relative lack of comprehensive research on scleroglucan recovery processes, we evaluated various alternatives to optimize and simplify gum recovery. We hypothesized that heating fermentation broth would increase gum recovery, since lysed cells could potentially release more intracellular and cell-bound scleroglucan. Various authors had previously used different alcohols for scleroglucan precipitation; therefore we compared the effectiveness of ethanol, iso-propanol and methanol with and without heat treatment[2,5,10,16,18,19,25-28]. In the first approach we used heat treatment of the 1:1 diluted whole broth to lyse the cells (Figure 2). In the second approach we heat treated the 1:1 diluted broth supernatant after cells were removed via centrifugation (Figure 3). In these trials we used one batch of cultured, filtered CCS medium, with three replications of 50 mL each processed for each method.

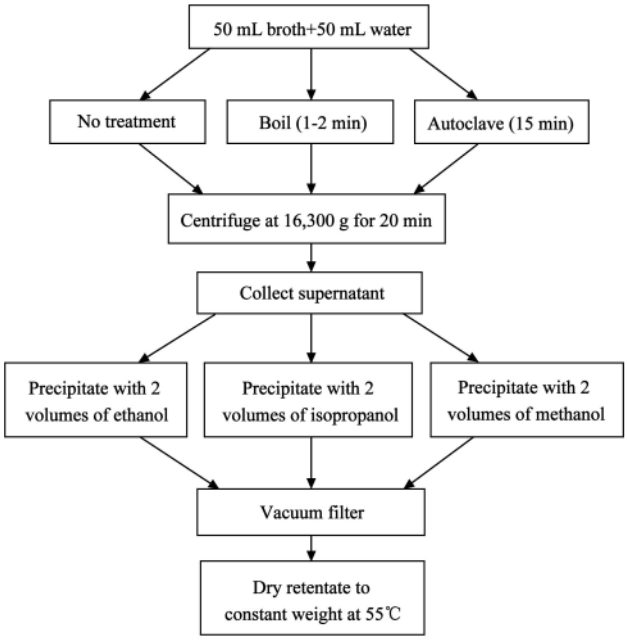

Figure 2 Method to evaluate cell lysis and alcohol precipitant on scleroglucan recovery

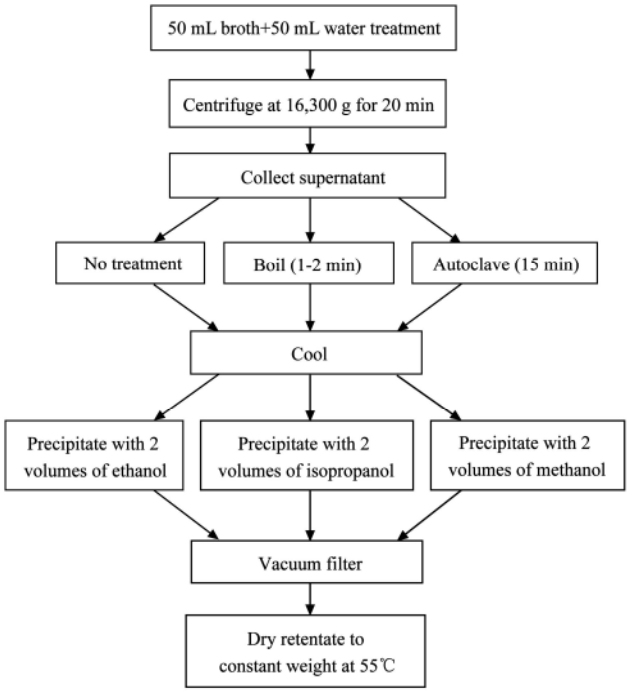

Figure 3 Method to evaluate thermal treatment and alcohol precipitant on scleroglucan recovery

2.5 Effect of cold treatment on scleroglucan recovery

During our normal culture maintenance procedures we refrigerated backup cultures, and sometimes froze samples of culture broth. We observed a drop in broth viscosity after these storage "treatments," and thus decided to evaluate the effect of cold treatment on scleroglucan recovery. To examine this effect, 50 mL samples of cultured, filtered CCS media (from both shake flask and bioreactor trials) were either processed immediately, refrigerated for 24 h at 4℃, or frozen for 24 h at –5℃ before recovery. Samples were then diluted with 50 mL of water and subjected to the more simplified recovery process shown in Figure 4.

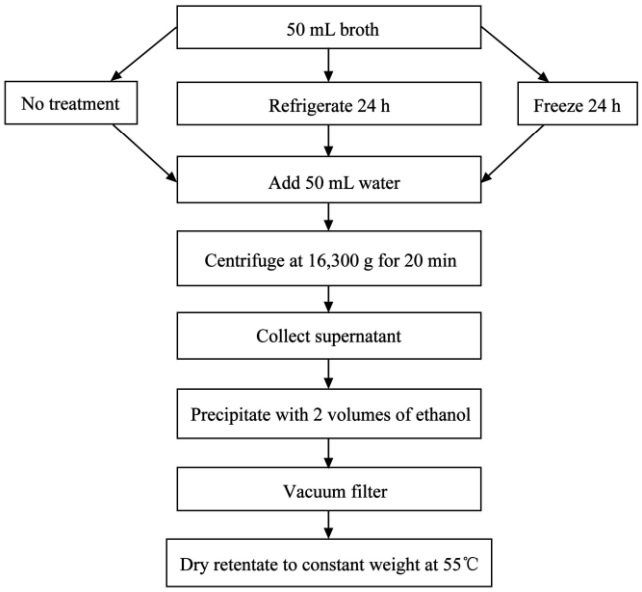

Figure 4 Method to evaluate cold treatment on scleroglucan recovery

2.6 Effect of diluting broth on scleroglucan recovery

Due to the high-viscosity of broth during the latter stages of scleroglucan production, it has been reported that dilutions of 1:1 to 1:3 (0.50–0.25) in water facilitate scleroglucan recovery[16,18,19,26,28]. We were interested in evaluating a broader range of dilution factors for scleroglucan recovery in the filtered CCS-based medium. Therefore after incubation, the broth was mixed with deionized water to obtain a total volume of 50 mL for dilutions ranging from 1 (undiluted) to 0.02. Recovery was performed via procedures in Figure 5.

2.7 Analytical methods

During scleroglucan production trials we used the following analytical methods to help ensure consistency from trial to trial. These data are provided in the article describing our efforts to develop a less expensive medium for scleroglucan production[20]. Sample pH was measured using an Accumet 950 pH/ion meter (Fischer Scientific). Broth samples were analyzed by HPLC for sugars, organic acids, ethanol, and glycerol (these data

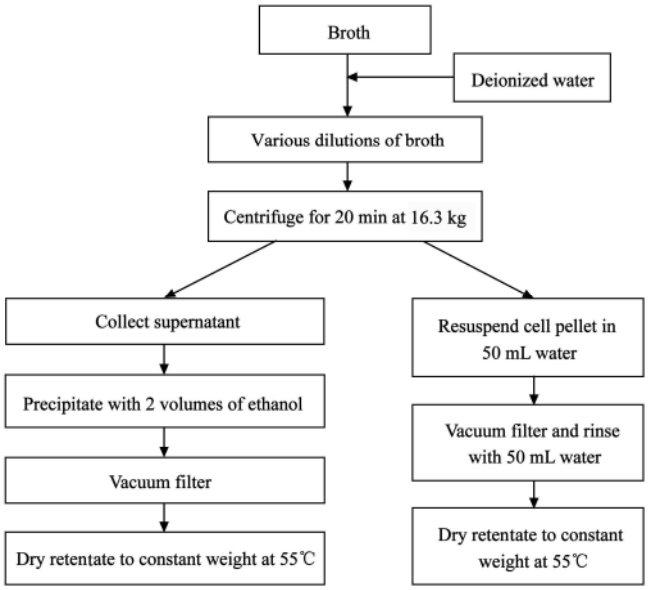

Figure 5 Method to evaluate effect of dilution on scleroglucan recovery

were used in other articles). Samples were filtered through a non-sterile 0.2 μm nylon membrane filter to remove cells and solids, placed in autosampler vials, and frozen until analysis. We used a Waters HPLC system with an Aminex®HPX-87H column operated at 58℃ with a 4 mM H_2SO_4 helium-degassed mobile phase at a flow rate of 0.6 mL/min. Peaks were detected using a refractive index detector.

3 Results and discussion

Scleroglucan recovery from culture broth typically involves first separating cells, and then precipitating scleroglucan from the supernatant. Removing the cells first allows a more precise quantification of scleroglucan[2,5,16,18,19,25-28]. In addition, health-care related applications of scleroglucan would require a cell-free form of the gum[9,12,14,15]. However, other applications (eg petroleum recovery) may not require separation of cells, hence reducing recovery costs[3]. We evaluated several steps in the typical scleroglucan recovery process to determine if they could be eliminated or modified to reduce costs. In each of these separate trials we used uniform batches of broth, to ensure that treatment effects could be differentiated. This was necessary because even though we used consistent conditions to culture *S. glucanicum*, there was variability in scleroglucan content between batches of broth.

3.1 Analysis of standard scleroglucan and cell biomass recovery method

To evaluate the effectiveness of the standard centrifugation, precipitation, and filtration method for scleroglucan and biomass fractionation (Figure 1), we tested cultured samples of both the modified Wang's and filtered CCS media. Table 1 shows the initial dry weight of 1 L of the fermented samples compared to the resulting fractions in cell pellet, scleroglucan and media solids (i.e. total filtrate). Over 96% of the scleroglucan was recovered from the initial centrifugation for both media types; meaning little scleroglucan was attached to the cells. Therefore in a commercial process it would not be worthwhile to conduct the cell washing and secondary centrifugation steps to recover this minimal amount of scleroglucan. Capital and operating expenses for the second centrifuge and the costs for recovering the ethanol precipitant from the second scleroglucan precipitation step would be prohibitive. Overall, the total solids recovery was high in both media types (97.8% for the Modified Wang's medium and 98.1% for the filtered CCS medium).

Table 1 Mass balance of standard scleroglucan and cell biomass recovery method

Fraction	Modified wang's medium dry weight/g · L^{-1}	Filtered CCS medium dry weight/g · L^{-1}
Fermented broth	27.0	36.5
Cell pellet	6.0	8.2
Scleroglucan from first centrifugation	10.1	14.2
Scleroglucan from second centrifugation	0.4	0.6
Total scleroglucan	10.5	14.8
Total Filtrate	9.9	12.8
Total solids recovery	26.4	35.8

3.2 Effect of cell lysis and alcohol precipitant on scleroglucan recovery

We conducted a series of trials to jointly evaluate 1) the effects of lysing cells in the whole culture broth using different heat treatments with 2) using different types of alcohols for scleroglucan precipitation of the supernatant after broth centrifugation (Figure 2). As controls we also tested these heat treatments of the supernatant after centrifugation (Figure 3). The goal was to maximize scleroglucan recovery using the simplest recovery method.

The results (Figure 6) show that no particular treatment was significantly better than the others. Even though a

single batch of batch of cultured, filtered CCS medium was used in this evaluation, we did observe a large amount of variability within replications. We believe this was due to multiple steps involved in the two recovery processes. In contrast to our hypothesis, the cells evidently contained little intracellular scleroglucan, thus lysing the cells had no beneficial effect on releasing intracellular or surface bound scleroglucan. This may have been due, in part, to the l44 h cultivation time we used to maximize scleroglucan production. Had we treated cells earlier in the production cycle, they may have contained more scleroglucan; however, yield would have suffered. Previous investigators have used methanol, ethanol, or isopropanol for scleroglucan precipitation, but there has been no direct side by side comparison[3,5,16,18,19,26-31]. Our results indicate that there was no statistically significant difference in performance. Based on these results, the simplest and cheapest method was to forgo the heat treatment and precipitate scleroglucan using ethanol. We selected ethanol because it would be available at the corn ethanol plants where the CCS used for the production medium would be available. Moreover, it would be convenient to recover and recycle the ethanol used for precipitation through the distillation systems present at these facilities.

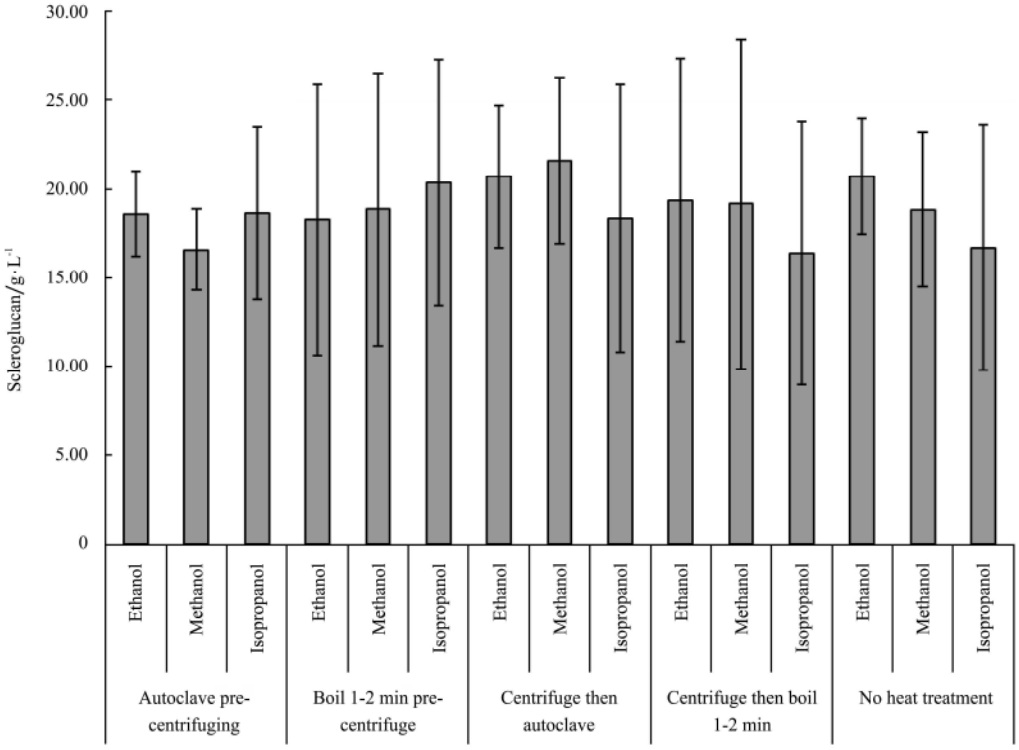

Figure 6 Effect of cell lysis and alcohol precipitant on scleroglucan recovery[a]

[a] Error bars represent standard deviation

3.3 Effect of cold treatment on scleroglucan recovery

To examine the effect freezing and refrigerating culture broth had on scleroglucan recovery, we processed filtered CCS medium from both shake flask and bioreactor trials by the procedure shown in Figure 4. In some treatments we again observed large variability with replications, likely caused by the multiple process steps. Samples processed immediately and those refrigerated 24 h did not show significant difference in scleroglucan recovery (Figure 7). Freezing drastically reduced scleroglucan recovery, perhaps due to disruption of scleroglucan's structure as a result of freezing. A more dramatic reduction occurred in the bioreactor samples that were frozen. We observed that mycelial filaments were much shorter and smaller in these trials compared to slightly pelleted, larger filaments in the shake flasks. This difference in mycelial form may have made the bioreactor-produced scleroglucan more susceptible to disruption by freezing.

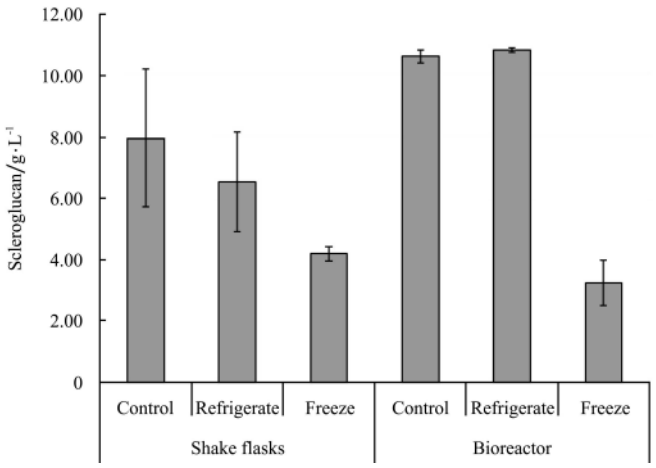

Figure 7 Effect of cold treatment on scleroglucan recovery[a]

[a] Error bars represent standard deviation

3.4 Effect of broth dilution on scleroglucan recovery

We evaluated a broader range of dilutions, using filtered CCS broth that had been incubated with *Sclerotium glucanicum* in the bioreactor. Our results of testing various dilutions (Figure 5), showed no recovery of scleroglucan at a dilution factor of 1.0 (undiluted), while the most effective separations occurred at dilution factors of 0.50 and 0.34. Others have also reported that dilution of cultured broth is necessary to achieve successful separation of cells and scleroglucan via centrifugation, with dilutions of 1:1 to 1:3 (dilution factor of 0.50 to 0.25) most commonly used[16,19,28]. Greater dilutions actually resulted in reduced scleroglucan recovery, with the further disadvantage of requiring significantly more ethanol for precipitation. Similar dilution results have been seen with recovery of the gum gellan[32,33].

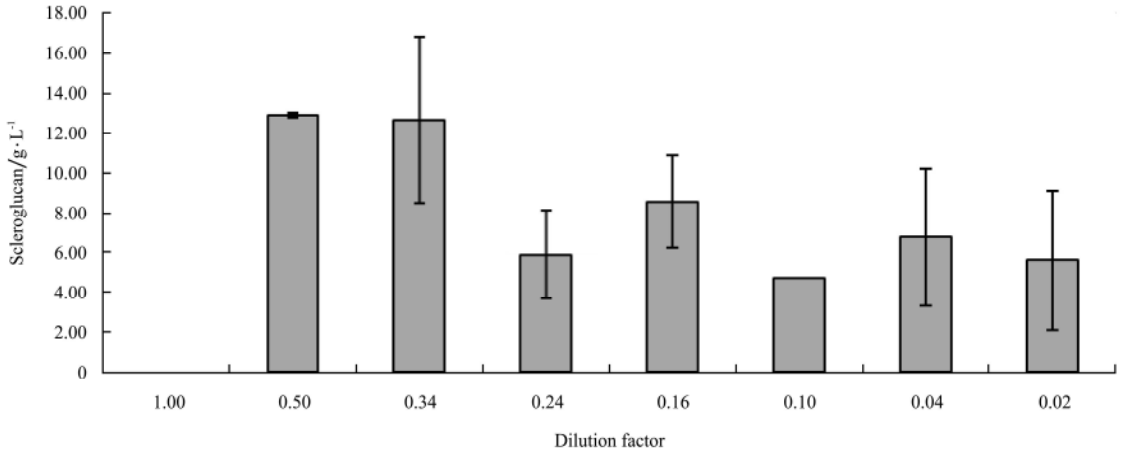

Figure 8 Effect of broth dilution on scleroglucan recovery.[a]

[a] Error bars represent standard deviation

4 Conclusions

Based on this work, we have established the optimum scleroglucan recovery method for commercial application that would be shown in Figure 5, using a dilution of 1:1 (dilution factor of 0.5) to facilitate separating the cell pellet from the scleroglucan-containing supernatant. We would recommend eliminating the step of washing the cell pellet, since that only increased scleroglucan recovery by 4%, while doubling the need for centrifugation and the amount precipitant needed. If scleroglucan was to be produced at an ethanol production facility (source of inexpensive CCS medium), then ethanol would be the lowest cost precipitant.

Due to its ability to form gels that are stable over a broad range of temperatures (0–120℃), pH (1–12.2), and salt concentrations (0–20%), scleroglucan has great potential for use in various applications[1,3,6] However production costs must be reduced for scleroglucan to find expanded markets in the pharmaceutical, food, industrial, and agricultural applications [11,12,14,15,16].

Acknowledgments

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