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Promoting pellet growth of *Trichoderma reesei* Rut C30 by surfactants for easy separation and enhanced cellulase production

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ABSTRACT

It is desirable to modify the normally filamentous *Trichoderma reesei* Rut C-30 to a pellet form, for easy biomass separation from the fermentation medium containing soluble products (e.g., cellulase). It was found in this study that this morphological modification could be successfully achieved by addition of the biosurfactant rhamnolipid (at ≥0.3 g/L) and the synthetic Triton X-100 (at ≥0.1 g/L) to the fermentation broth before the cells started to grow actively. Thirteen other surfactants tested were not as effective. Furthermore, the added rhamnolipid and Triton X-100 increased the maximum cellulase activity (Filter Paper Units) produced in the fungal fermentation; the increase was 68 ± 7.8% for rhamnolipid and 73 ± 12% for Triton X-100. At the concentrations required for pellet formation, rhamnolipid had negative effect on the cell growth: with increasing rhamnolipid concentrations, the growth rate decreased and the lag-phase duration increased linearly. Triton X-100 caused no significant differences in growth rate or lag phase.

1. Introduction

Microorganisms have been used to produce various useful materials, including the cultivation of fungi for the purpose of food, antibiotic and enzyme production [1,2]. One of the well-studied fungi is *Trichoderma reesei* Rut C-30, a hyper-cellulase producing strain. The Rut C-30 strain is known to produce the highest cellulase activity at pH 4.5, while the optimal pH for xylanase production and growth is 6.0 [3,4]. Various enzyme inducing agents have been studied, with cellulose, oligosaccharides (hydrolysis intermediates) and sophorose providing the greatest expression potential and lactose to a lesser extent [5–8]. The use of cocultures of *Candida bombicola* and *T. reesei* to produce sophorolipids *in situ*, as the source of sophorose for inducing cellulase production, has also been reported [9].

Aside from process conditions and inducing agents, some surfactants (e.g., Tween-80, Tween-60, and Tween-20) have been found to improve the release of extracellular proteins and increase activities of cellulase and xylanase enzymes from *T. reesei* and *Trichoderma viride* [10–12]. It is believed that these surfactants can increase the enzyme transport across cell membrane [11], [13–15].

While many studies have provided insight into the optimal growth conditions, one industrially important aspect for separation operations is the mycelial biomass morphology. The naturally filamentous morphology of *T. reesei* causes a significant increase in broth viscosity and the biomass becomes difficult to separate from the liquid medium. Therefore, formation of compact mycelial pellets is desirable for the purpose of easy cell separation. Many factors can affect fungal morphology in submerged fermentation but generalization and prediction of behavior caused by these factors on different species at different culture conditions is difficult. It has been demonstrated that when spore suspensions are used as inocula, the inoculation conditions can affect the morphology in some species, with generally lower inoculation of spores promoting the formation of pellets and higher inoculation concentrations yielding to flocs [16]. For instance, this effect was well studied for *Aspergillus niger* confirming that the pellet forming tendency decreases with increasing inoculum spore concentration [17]. Yet, regardless of the spore concentration used for inoculation, the addition of 0.5 g/L of Tween-80 could inhibit the formation of pellets of *T. reesei* Rut C-30 [14]. The carbon sources/additives used in the medium can also affect pellet formation. For example, various strains of *A. niger* were shown to grow as free mycelia on lactose as a substrate but the same species could form pellets when glucose was added to the medium [18]. Similarly, the addition of sorbose with *T. reesei* QM9414 was found to induce a clear shift from filamentous to compact pellet form [19]. The addition of carboxypolymethylene or carboxymethylcellulose to cultures of *Aspergillus fumigatus* also promoted smaller discrete pellets over larger globular formations [20].

Surfactants are a special group of additives that can also affect fungal morphology. Previous work has described the role of

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surfactants on the morphological structure of *Penicillium*, particularly about how the added compounds could cause or inhibit pellet formation [21–23]. It has also been reported that the addition of Tween-40 to a *Trichoderma harzianum* shake flask culture might promote pellet formation [24]. For *T. reesei* Rut C-30 a study has indicated that Tween-80 might inhibit its pellet formation [14]. Although Tween-80 may inhibit pellet formation, it was shown that a 50 mM pH 4.8 buffer of citrate or phthalate will promote *T. reesei* to form compact pellets while succinate and formate buffers produces soft pellets, and an unbuffered system yields only dispersed morphology. In all cases the pellet formation was diminished with 100 mM buffer concentration or non-optimal pH, indicating that the concentration, chemical type and pH can all be significant factors to pellet formation [25]. There have been no other known studies on the surfactant effects on the morphology of *T. reesei*.

In this work, pellet formation of *T. reesei* Rut C-30 (in the common medium containing 0.2 g/L Tween-80) is reported to be effectively promoted by the addition of some surfactants. The effects of these surfactants on cell growth and cellulase production in a submerged fermentation process are also discussed.

2. Materials and methods

2.1. Surfactants

Triton X-100 (Triton), Tween-80, Tween-20 and Span-80 were obtained from Sigma–Aldrich (St. Louis, MO, USA). A concentrated solution of rhamnolipid mixture was purchased from EchoChem (Delia, AB, CA). Pluronic® surfactants were obtained from BASF (Parsippany, NJ, USA).

2.2. Microorganism

T. reesei Rut-C30 (NRRL 3469) was obtained from the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection (also known as the NRRL Collection). The culture was maintained on slants of potato dextrose agar (30 g/L, Sigma, P2182) at 4 °C and sub-cultured every 4 weeks. Inoculum was prepared by aseptically adding three loops of cells from a mature slant to potato dextrose broth (24 g/L, Sigma P6685), and incubating for 3 days at room temperature on a magnetic stir plate set to approximately 300 rpm. This provided an entirely filamentous culture, at the late exponential growth phase, for inoculation of the test systems. All systems were inoculated at 10% (v/v). This inoculation technique provides a high concentration of actively growing hypha in an attempt to avoid pellet formation caused when inoculating with a low concentration of spores.

2.3. Fermentation medium

Unless otherwise specified, the chemicals used were from either Fisher Scientific (Fairlawn, NJ, USA) or Sigma–Aldrich (St. Louis, MO, USA). All cultures were grown with a modified Mandels and Webber medium containing (g/L): $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $MgSO_4{\cdot}7H_2O$ 0.3, $CaCl_2{\cdot}2H_2O$ 0.4, urea 0.3, proteose peptone (Remel) 1.0, Tween-80 0.2, $FeSO_4{\cdot}7H_2O$ 0.005, $MnSO_4{\cdot}H_2O$ 0.0016, $ZnSO_4{\cdot}7H_2O$ 0.0014, $CoCl_2$ 0.002, and lactose 10.0 [10]. The unadjusted pH was 5.5 after autoclaving.

2.4. Fermentation setup

Test cultures were grown in 50 mL volume in 250 mL flask at 28 °C in a Que Orbital shaker (Model 4703, Parkersburg, WV, USA) at 250 rpm without pH control, initially at 5.5, for 7 days. Triton or rhamnolipid was tested at the following concentrations (g/L): 0.0, 0.1, 0.3, 0.6, 0.8, 1.0 and 1.5. Cultures were followed for 7 days and visually checked for bulk morphology. Periodic samples (2 mL) were taken aseptically in a laminar flow hood (Forma Scientific Model 1839, Marietta, OH) every 24 h to measure mycelial biomass, cellulase enzyme activity, sugar concentration and residual surfactant concentration.

2.5. Mycelial biomass estimation

The intracellular protein concentration was measured and then converted to the mycelial dry-weight concentration according to a previously established correlation [26]. The procedure is briefly described in the following: The culture flask was manually mixed to suspend the biomass and simultaneously a 1.0 mL sample was collected. The sample was centrifuged at 9300 × *g* for 10 min to obtain a pellet. The supernatant was collected for further processing. The pellet was re-suspended and washed twice with de-ionized water. After each wash step, the biomass was centrifuged and the water discarded. Following the last wash step, all samples were frozen at −20 °C while waiting testing. To release the intracellular proteins, an unfrozen pellet was suspended in 1.0 mL of 0.2 M sodium hydroxide and heated at 100 °C for 20 min. Following cooling, the digested sample was centrifuged at 5900 × *g* for 10 min to remove cell debris; the supernatant was determined for protein concentration by using the Bio-Rad Protein Assay Kit (Bio-Rad, Kit# 500-002, Hercules, CA, USA). The protein assay was calibrated with bovine serum albumin solutions as standards. The calculated protein concentration was converted to equivalent mycelium dry weight by a previously established correlation [26].

2.6. Sugar concentration and cellulase activity estimation

Reducing sugar concentrations were measured using a non-specific 3,5-dinitrosalicylic acid (DNS) test method [27]. Cellulase activities, in terms of Filter Paper Units (FPU), were estimated using the method proposed by Ghose for dilute concentrations [26,27]. For the FPU activity determination, 100 μL of supernatant was mixed in 1.4 mL of a pH 4.8 and 0.05 M sodium citrate buffer in a tall 25 mL test tube and incubated at 50 °C for 1 h in an orbital reciprocating water bath (Boekel Grant, ORS 200) set at 50 rpm. In this test, the enzyme sample contained a 1 cm × 6 cm strip of Whatman #1 filter paper, while the blank was incubated without the filter paper substrate. The reaction was stopped with the addition of 3 mL DNS reagent and heated to 100 °C for 5 min for color development. Absorbance at 595 nm was correlated to a standard glucose concentration curve.

2.7. Triton X-100 concentration measurement

Triton concentrations were determined by the reverse phase high-performance liquid chromatography (HPLC) using a Hewlett-Packard LC1100 series HPLC (Palo Alto, CA, USA) fitted with a G1315A diode array detector, a temperature controlled auto-injector, and a Waters Symmetry C18 column (Milford, MA, USA) operating at 24 °C. The sample injection volume was 20 μL. A quaternary pump provided the following water–acetonitrile sequence to isolate the Triton: 4 min hold at 40:60, 3 min ramp to 10:90, 2 min hold at 10:90, 6 min ramp to 40:60 and a 15 min hold at 40:60. The flow rate of mobile phase was 0.5 mL/min. The Triton concentration was correlated to the height of a 200-nm absorbance peak at the retention volume of 8.05 mL. Samples were prepared for analysis by diluting the supernatant from centrifuged culture broth with equal volume of methanol and then centrifuging at 10,000 × *g* to remove insoluble components [28]. The methanol–supernatant mixture was stored at 4 °C while in the auto-injector to prevent microbial growth. Standards were prepared in a similar method, neglecting the final centrifugation.

2.8. Rhamnolipid concentration measurement

Rhamnolipid concentrations were determined by a modified Orinoco method [29]. In a disposable polypropylene micro centrifuge tube, a 50 μL sample was mixed with 100 μL of 10 mM phosphate buffer, pH 2.5, and extracted at room temperature with 1.0 mL of ethyl acetate for 24 h in a shaker at 250 rpm (Que orbital shaker, Model 4703, Parkersburg, WV, USA). The ethyl acetate-aqueous mixture was centrifuged at 8000 rpm for 10 min to accelerate phase separation. Into a clean glass test-tube, 0.5 mL of the ethyl acetate phase was collected and allowed to evaporate. The dry rhamnolipid residue was re-dissolved in 0.85 mL of a 0.05 M sodium bicarbonate solution added into the glass test tube. The tube and solution was then cooled in an ice water bath before addition of 1.65 mL refrigerated anthrone reagent (2 g/L anthrone in concentrated sulfuric acid at 4 °C) [30]. Vials were sealed using polytetrafluoroethylene lined caps and heated at 95 °C for 16 min. After cooling to room temperature, absorbance was measured at 635 nm using a spectrophotometer (Model UV-1601, Shimadzu Corporation, Columbia, MD). Calibration standards were obtained with pure rhamnose dissolved in the sodium bicarbonate solution and processed by the same procedure following the addition of the anthrone reagent. A correlation factor of 2.87 was used to convert the measured rhamnose concentration to the equivalent rhamnolipid concentration.

3. Results

3.1. Surfactant screening

A series of surfactants were screened for their effects on the morphology of *T. reesei* Rut C-30. In a sequence of shake flask studies, 1.0 g/L of each tested surfactant was added to the fresh medium before inoculation. A visual observation of cell morphology was used to estimate the response. These visual observations are summarized in Table 1. At 1 g/L Brij-52, Tween-80, and a majority of the non-ionic Pluronic surfactants did not affect the morphology. However, Span-80 and Pluronic L31, F77, and a 50/50 mixture of F77 and L61 increased the filamentous nature of the fungus. Tween-20, Pluronic P65 and F88 showed slight tendencies to increase flocculation of the cells. Yet, these surfactants were unable to produce true "pellets." The sodium dodecyl sulfate (SDS) was inhibitory to the cell growth at the 1 g/L concentration tested. However, as shown by