Food Chemistry 224 (2017) 139–143

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Effects of nonionic surfactants on pellet formation and the production of β-fructofuranosidases from *Aspergillus oryzae* KB

Masahiro Kurakake [a,b,*], Saya Hirotsu [b], Miyuki Shibata [b], Yuta Takenaka [b], Taiki Kamioka [b], Tatsuya Sakamoto [b]

[a] Department of Marine Biotechnology, Fukuyama University, 1-985 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan
[b] Department of Life and Nutritional Science, Fukuyama University, 1-985 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan

ARTICLE INFO

*Article history:*
Received 21 July 2016
Received in revised form 17 November 2016
Accepted 19 December 2016
Available online 21 December 2016

*Keywords:*
Pellet
Surfactant
Fructosyltransferase
Transfructosylation
Tween
*Aspergillus*

ABSTRACT

*Aspergillus oryzae* KB produces two β-fructofuranosidases (F1 and F2). F1 has high transferring activity and produces fructooligosaccharides from sucrose. Mycelial growth pellets were altered by the addition of Tween 20, 40 and 80 (HLB = 16.7, 15.6 and 15.0, respectively) in liquid medium cultures to form small spherical pellets. The particle size of the pellets decreased with the HLB value, which corresponds to an increase in surfactant hydrophobicity. Selective F1 production and pellet size were maximized using Tween 20. Adding polyoxyethylene oleyl ethers (POEs) with various degrees of polymerization (2, 7, 10, 20 and 50: HLB = 7.7, 10.7, 14.7, 17.2 and 18.2, respectively) was investigated. A minimum mean particle size was obtained using a POE with DP = 10, HLB = 14.7. The POE surfactants had little effect on the selective production of F1. The formation of filamentous pellets depended on the surfactant HLB value, and F1 enzymes were produced most efficiently using Tween 20.



## 1. Introduction

Fructooligosaccharides (FOSs)—such as 1-kestose, nystose and fructosyl-nystose—are used as food ingredients because they have beneficial health effects, such as improved growth of bifidobacteria in the intestinal flora. FOSs are produced by β-fructofuranosidases with high transferring activity (Ut) (Dominguez, Rodrigues, Lima, & Teixeira, 2014). Many β-fructofuranosidases have both Ut and hydrolyzing activity (Uh), and the ratio of Ut/Uh varies depending on the origin of the enzyme. The two types of β-fructofuranosidase (named F1 and F2) from *Aspergillus oryzae* KB have been isolated, and their enzymatic properties were investigated (Kurakake et al., 2008). F1 produced 1-kestose, nystose and fructosyl-nystose from sucrose through transfructosylation activity, whereas F2 hydrolyzed sucrose to mainly glucose and fructose.

The KB strain forms filamentous pellets containing enzymes in liquid culture. Pellet formation is an advantageous fungal morphology for the production and application of enzymes because a pellet is easy to remove from the reaction mixture and use immediately as an immobilized enzyme in a flow reactor. Many immobilized enzymes have been studied for the continuous production of FOSs (Gonçalves, Jorge, & Guimarães, 2015; Kurakake et al., 2010; Lin & Lee, 2008; Lorenzoni, Aydos, Klein, Rodrigues, & Hertz, 2014). However, they needed the processes which isolates enzyme from culture broth and immobilizes it on carrier. It would be beneficial in terms of cost to use the filamentous pellet as an immobilized enzyme.

Ungerminated spores aggregate to form nucleators for pellet growth at an early stage of a liquid culture. Adherence among germinated spores and hyphae interactions grow the pellet structure (Lin, Grimm, Wulkow, Hempel, & Krull, 2008; Priegnitz et al., 2012). The pellet development is influenced by various culture conditions, such as the initial pH value, medium composition, additives and aeration (Grimm, Kelly, Volkerding, Krull, & Hempel, 2005; Znidarsic, Komel, & Pavko, 2000). Addition of a nonionic surfactant, such as Tween, in the culture medium influenced fungi pellet formation. Tween 80 enhanced the mycelial growth and exopolysaccharide production in the submerged fermentation of *Pleurotus tuber-regium* and stabilized the structure of the mycelial pellets (Zhang & Cheung, 2011). The pellet formation of *Trichoderma reesei* Rut C-30 was inhibited by the addition of Tween 80 in the fermentation medium, but improved cellulase production (Domingues, Queiroz, Cabral, & Fonseca, 2000). In KB strain, it is interesting to investigate an influence of the pellet size on the selective production of the F1 enzyme which produces FOSs through the transglycosylation. F2 enzyme is undesired because the hydrolyzing activity decreases the production of FOSs.

* Corresponding author at: Department of Marine Biotechnology, Fukuyama University, 1-985 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan.
*E-mail address:* kurakake@fubac.fukuyama-u.ac.jp (M. Kurakake).

http://dx.doi.org/10.1016/j.foodchem.2016.12.054
0308-8146/

In the present study, nonionic surfactants (Tween and polyoxyethylene oleyl ethers POEs) were added to the liquid culture medium of *Aspergillus oryzae* KB and their effects on pellet formation and production of F1 and F2 enzymes were investigated. The HLB (hydrophile lipophile balance) value of nonionic surfactant was changed for the examinations.

## 2. Materials and methods

### 2.1. Materials

1-Kestose, nystose and fructosyl-nystose were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). The *A. oryzae* KB strain was separated from malted rice used for the production of the traditional Japanese liquor "sake". Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 40 (polyoxyethylene sorbitan monopalmitate) and Tween 80 (polyoxyethylene sorbitan monooleate), which have HLB (hydrophile lipophile balance) values of 16.7, 15.6 and 15.0, respectively, were purchased from Sigma–Aldrich (St Louis, MO, USA). Polyoxyethylene oleyl ethers, POEs 2, 7, 10, 20 and 50, which have HLB values of 7.7, 10.7, 14.7, 17.2 and 18.2, respectively, were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The numbers indicate the degrees of polymerization of the polyoxyethylene. All other chemicals were of reagent grade and commercially available.

### 2.2. Production of β-fructofuranosidases in liquid culture

*A. oryzae* KB strain was cultured in a liquid medium (30 ml) containing 1% sucrose, 0.5% yeast extract and 0.2% $Na_2HPO_4{\cdot}12H_2O$ in a 100-ml Erlenmeyer flask at 30 °C and 150 rpm for 48 h. Tween and POE nonionic surfactants were added to the medium at 1.0% and 0.5% concentrations, respectively. Although the inoculum of the strain spores was $4 \times 10^6$ spores for experiments with Tweens, it was decreased by one tenth (relative to $4 \times 10^5$ spores) for those with POEs in order to observe pellet formation more clearly by preventing an overproduction of pellets. The culture broth was transferred to a 9-cm petri dish and photographs were taken to measure the diameter of the mycelial pellets. The mycelial pellets were separated by decantation in a 50-ml polystyrene conical tube and squeezed with a spatula. The pellets were washed with distilled water five times, and 50 mM acetate buffer (pH 5) was added to the initial volume. The mycelial pellets were homogenized in a 50-ml tube using a Bio-mixer (Nihonseiki Kaisha Ltd., Tokyo, Japan). Enzyme activities were determined for the homogenized suspensions in 50 mM acetate buffer (pH 5). Two times of experiments were done and error values (standard deviation) were calculated.

### 2.3. Determination of β-fructofuranosidase activity

One percent (w/v) sucrose was incubated with the enzymes at pH 5 for 10 min at 40 °C. The reaction (volume, 1 ml) was stopped by the addition of 1 M $Na_2CO_3$. Glucose produced in the reaction mixture was determined by the glucose oxidase/peroxidase method using a Glucose CII-Test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). Enzyme activity was defined as the amount of enzyme required to produce 1 μmol of glucose from the sucrose in 1 min.

### 2.4. Determination of hydrolyzing (Uh) and fructosyl-transferring (Ut) activities

Uh and Ut were measured at a high concentration of sucrose (20% w/v). The sucrose was incubated with the enzymes at pH 5 and 40 °C for 2 h. The enzyme sample was diluted to 1 U/ml β-fructofuranosidase activity and 0.1 ml was used in the reaction (working volume: 0.5 ml). The reaction was stopped by incubating for 10 min in boiling water and the reaction mixture subjected to HPLC analysis after dilution and filtration through a 0.22 μm membrane filter. The two enzymatic activities were calculated from the amounts of glucose (G) and fructose (F) produced. Total activity (Utot = Ut + Uh), corresponding to degradation of sucrose, was defined as the amount of enzyme that could produce 1 μmol of glucose from the sucrose. Values of Ut and Uh were obtained by multiplying Utot by the transfer ratio (G−F)/G and the hydrolysis ratio (F/G), respectively. Ut and Uh were defined as the amount of enzyme that could hydrolyze 1 μmol of sucrose and transfer 1 μmol of fructosyl residues, respectively.

### 2.5. HPLC analysis of sugars

Glucose and fructose in the reaction mixtures were analyzed by HPLC using the following conditions: column, 250 mm × 4.6 mm i.d. NH2P-50 (Asahi Chemical Industry Company, Limited, Kawasaki, Kanagawa, Japan); mobile phase, acetonitrile–water (70:30 v/v); flow rate, 1.0 ml/min; pump, Tosoh model CCPS (Tosoh Co., Tokyo, Japan); detector, Tosoh model RI8020 differential refractive index monitor (Tosoh Co., Tokyo, Japan).

## 3. Results and discussion

*A. oryzae* KB was cultured in 1% sucrose medium with 1% Tween 20, 40 and 80. Mycelial growth pellets were distorted and large without Tween, and formed small and spherical pellets in the presence of the surfactants (Fig. 1). The particle size of the pellets was dependent on the type of Tween. The property of the nonionic surfactants was established using the HLB (hydrophile lipophile balance) value. A higher value means a more hydrophilic and less hydrophobic property. The HLB is controlled by the alkyl chain length of the fatty acids bound to the polyoxyethylene chain. The mean particle size of the mycelial pellets formed in the presence of Tween 20 was 3.4 ± 0.4 mm and larger than those formed with Tween 40 (3.1 ± 0.7 mm) and 80 (2.4 ± 0.6 mm) (Table 1). The mean particle sizes of the pellets corresponded to the HLB values of the Tween surfactants, and decreased with decreasing HLB value. Ungerminated spores aggregate and form nucleators for pellet growth as an initial period in the formation of pellets (Lin et al., 2008; Priegnitz et al., 2012). It seems that the Tween surfactant causes the formation of smaller size pellets because the surfactants inhibit the aggregation of ungerminated *A. oryzae* KB spores. The inclusion of Tween 80 in the culture medium of fungus *Cordyceps sinensis* inhibited the aggregation of mycelia and led to the formation of smaller pellets (Liu & Wu, 2012).

The growth of the mycelium was improved and increased the pellet mass using a surfactant in the liquid culture because nutrition and air were supplied efficiently in the liquid phase (Table 1). Tween 80 increases mycelia membrane permeability and enhances mass transfer of nutrients into the pellets (Znidarsic et al., 2000). It is also important that the surface area of the pellets is increased by the formation of small and spherical particles.

A Tween surfactant with a low HLB value, which is highly lipophilic, decreases the exposure of hydrophobic ungerminated spore surfaces to the liquid culture. This action may disturb the aggregation of spores through hydrophobic interactions and result in decreased pellet particle size (Dynesen & Nielsen, 2003). The pellets grow to a large size by adherence of germinated spores and hyphae interactions. The pellets grown on a rotary shaker at 150 rpm were smooth and spherical. Tween surfactants with greater hydrophobicity (lower HLB values) seemed to disturb the pellet growth more. This finding suggests that adjustment of the pellet size is controlled by the HLB value of the nonionic surfactant.