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Acetylation and deacetylation for sucralose preparation by a newly isolated *Bacillus amyloliquefaciens* WZS01

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Sucralose is a non-nutritive artificial sweetener used in a broad range of foods and beverages. In the present study, *Bacillus amyloliquefaciens* WZS01 was isolated, identified, and used as a catalyst both in regioselective acylation and deacetylation for sucralose preparation. Bacterial cells were immobilized on polyurethane foam and utilized to synthesize sucrose-6-acetate regioselectively. The yield of sucrose-6-acetate was >95% with 60 mM sucrose after 22 h of reaction. Free cells could hydrolyze 75 mM sucralose-6-acetate to produce sucralose with >99% yield after 24 h of reaction. *B. amyloliquefaciens* WZS01 could be considered a potential biocatalyst for sucralose preparation.

[**Key words:** *Bacillus amyloliquefaciens*; Sucralose; Sucrose-6-acetate; Acetylation; Deacetylation]

Sucralose is a non-nutritive artificial sweetener, which is approximately 600 times as sweet as sucrose (1). The commercial success of sucralose-based products is attributed to their advantages over other low-calorie sweeteners in terms of taste, stability, and safety. Sucralose preparation involves complex processes, including sucrose-6-acetate synthesis, chlorination, and deacetylation. The synthesis of sucrose-6-acetate, which is an intermediate product, is a key step in sucralose preparation (2). However, the reaction conditions in chemical methods are complicated, such as the methods involving sucrose alkyl 4,6-orthoacylates (3) or dibutyltin oxide (4). As such, enzymes have been used to synthesize sucralose because of several advantages, including specificity and efficiency. Nevertheless, the types of enzymes catalyzing the highly regioselective acylation of sucrose are relatively few. For instance, a 32.9% yield of 6-*O*-acetyl sucrose is obtained by using lipase P Amano (*Pseudomonas* spp.), but large quantities of 4′,6-di-*O*-acetylsucrose are also generated (5). Lipase from *Thermomyces lanuginosus* is also used efficiently for the regioselective esterification of 6-OH in sucrose to produce 6-*O*-lauroylsucrose (6) and sucrose-6-acetate (7). A yield of 87.46% sucrose-6-acetate with 2% sucrose has been the highest value obtained after optimization and is accomplished via cross-linked enzyme aggregates of Lipozyme TL 100 L (8).

Sucralose synthesis also involves the removal of a 6-acetyl group from sucralose-6-acetate. This step is generally conducted in methanol solution with sodium methoxide or sodium hydroxide as a catalyst, which introduces a large number of sodium ions into the reaction system. As such, numerous downstream processing and product purification steps are required. These drawbacks can be resolved through sucralose-6-acetate deacetylation with enzymatic or whole-cell biocatalysis. Ratnam et al. (9) reported that a maximum of 95% deacetylation was achieved by using free or immobilized enzymes. Chaubey et al. (10) used two indigenous strains to hydrolyze sucralose-6-acetate and thus produced sucralose with 100% purity. These methods are environmentally friendly and do not involve hazardous reagents.

In this study, *Bacillus amyloliquefaciens* WZS01 was isolated and identified. As shown in Fig. 1, the cells of WZS01 immobilized on polyurethane foam (PUF) were used to synthesize sucrose-6-acetate and free cells were used to hydrolyze sucralose-6-acetate to sucralose. To our knowledge, the use of the same whole-cell catalysts both in regioselective acylation and deacetylation for sucralose preparation has remained unexplored. Influencing factors, such as reaction temperature and pH, were also investigated. Our study revealed that the bacterial strain WZS01 could be considered a potential biocatalyst for sucralose preparation.

MATERIALS AND METHODS

Materials Sucrose-6-acetate, sucralose-6-acetate, and sucralose were purchased from Sigma. Tributyrin, 4 Å molecular sieve, and tertiary butanol were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All other chemicals were of analytical reagent grade.

Screening and isolation of sucrose-6-acetate-producing organisms Soil samples were collected from various oil-contaminated environments located in Zhejiang Province, China. Microbes producing lipase were enriched by inoculating 0.1 g of soil sample into 20 mL olive oil medium (3.0% olive oil, 0.5% Tween-80, 0.50% $(NH_4)_2SO_4$, 0.35% K_2HPO_4, 0.10% KH_2PO_4, 0.25% NaCl, and 0.05% $MgSO_4 \cdot 7H_2O$) in flasks. The sample was incubated in an orbital shaker at 30 °C and 150 rpm for 2 days. The samples of batch culture were streaked on tributyrin agar plates by using bromothymol blue as a pH indicator. The microorganisms that appeared yellow after 24 h of incubation at 30 °C were further purified by repeated streaking and then cultured.

The reactants for the selection of the microbes catalyzing sucrose-6-acetate synthesis contained 60 mM of sucrose, 10 mg of lyophilized cells, and 700 mM of vinyl acetate in 1 mL of tertiary butanol/dimethylformamide (DMF) (v/v, 4:1). The

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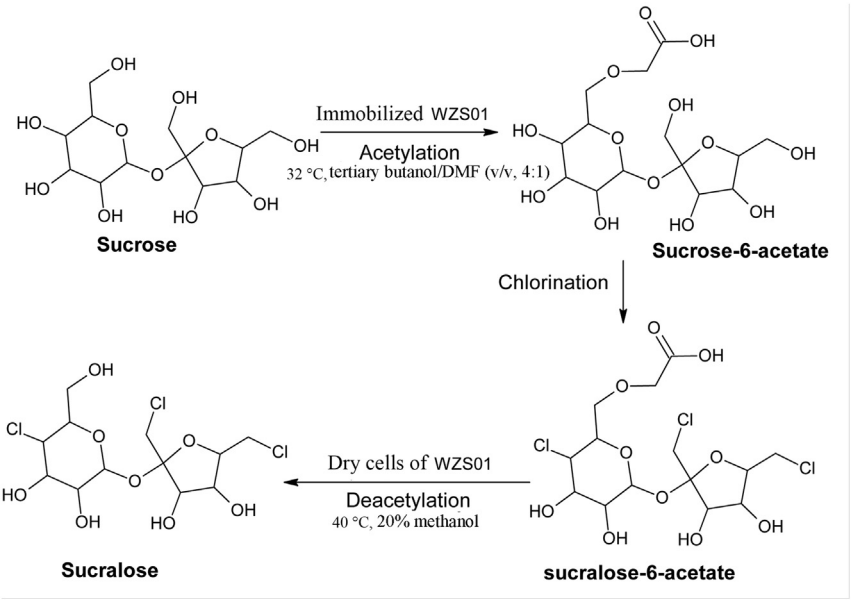

FIG. 1. The cells of *B. amyloliquefaciens* WZS01 catalyzed acetylation and deacetylation for sucralose preparation.

reaction was performed at 30 °C for 30 min with a rotation rate of 150 rpm. The esterification activity was measured by the determination of released acetaldehyde through colorimetry via 3-methyl-2-benzothialinone derivatization (11). The strains producing more acetaldehyde were selected and the reaction mixture was determined by high performance liquid chromatography (HPLC).

Strain identification After total DNA was extracted from the isolated strain WZS01, the primers 5′-CAGAGTTTGATCCTGGCT-3′ and 5′-AGGAGGTGATCCAGCCGCA-3′ were used to amplify 16S rDNA fragment (12). The primers 5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3′ and 5′-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3′ were utilized to amplify *gyrB* fragment (13). The gene *gyrB* encoding gyrase β subunit has been widely used as a phylogenetic marker (14). The nucleotide sequences were aligned with the sequences retrieved from GenBank using CLUSTALX 2.0. Phylogenetic trees based on 16S rDNA and *gyrB* sequences were constructed via a neighbor-joining algorithm in MEGA 6.0.

Cultivation conditions and polyurethane foam immobilization WZS01 was cultured in a sterile medium containing 2.5% glucose, 1% peptone, and 0.1% yeast extract. PUF with a density of 10 kg m^{-3} was cut into 1 cm^3 cubes, boiled in deionized water, and dried. Afterward, 0.4 g of PUF cubes were added into 50 mL of medium. The flasks were shaken for 18 h at 150 rpm and 30 °C. The cultures were harvested by squeezing the PUF, and the remaining parts of liquid cultures were

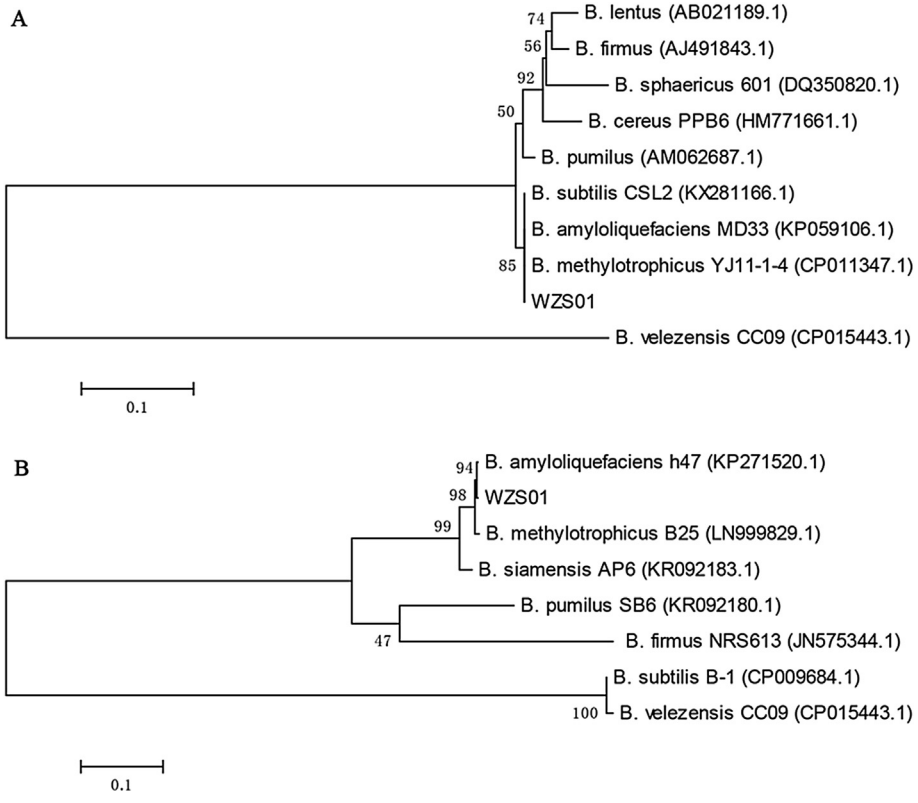

FIG. 2. Phylogenetic tree displaying the relationship between WZS01 and other *Bacillus* species on the basis of 16S rDNA (A) and *gyrB* (B) gene sequence analyses. The numerals show the significance of the branching order as determined by bootstrap analysis (bootstrap values of >85% were considered significant).

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