Journal of Bioscience and Bioengineering
VOL. 127 No. 3, 326–332, 2019

www.elsevier.com/locate/jbiosc

Highly efficient deproteinization with an ammonifying bacteria *Lysinibacillus fusiformis* isolated from brewery spent diatomite

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Received 29 April 2018; accepted 7 August 2018
Available online 15 September 2018

To explore a new method for bio-regeneration of high-protein brewery spent diatomite, an ammonifying bacteria (BSD1) was screened out from it and identified as *Lysinibacillus fusiformis*. The protein degradation characteristics of BSD1 was studied with rice protein as the sole nitrogen source. Maximum protein degradation activity was obtained when BSD1 was inoculated with an inoculum of 5% into a medium with glucose as carbon source and initial pH value of 7.0 and incubated at 30°C for 48 h. In this optimal condition, protein concentration decreased from 156.8 mg/L to 19.2 mg/L, and protein degradation efficiency of BSD1 reached 88%. Free amino acid analysis showed that the content of Phe, Tyr, Pro, Ala, Lys, Thr and His increased in protein degradation process. After degradation, NH_4^+–N concentration producing in medium supernatant reached 232.2 mg/L. These results indicated the strain BSD1 could transform proteins into free amino acids and eventually convert them to ammonium or ammonia. Furthermore, strain BSD1 could also be used for deproteinization of brewery spent diatomite and 51% of proteins in spent diatomite were degraded. After biological deproteinization the specific surface area and total pore volume of diatomite improved obviously. These results illustrated that the application of strain BSD1 for bio-regeneration of high-protein brewery spent diatomite was efficient and feasible.

[**Key words:** Brewery spent diatomite; Ammonifying bacteria; Protein degradation; Biological regeneration; *Lysinibacillus fusiformis*]

As the fifth most consumed beverage in the world apart from tea, carbonates, milk and coffee, beer is a popular alcoholic drink with an average consumption of 23 L per person per year (1,2). Beer production is a multi-step process mainly involving mashing, fermentation, filtration, and filling (3). The industrial production of most beers requires a filtration step as a part of downstream treatment process (4). Beer filtration combining with the addition of adsorbent agents is a most widely used technique and different filter aids may be used in this process, such as activated carbon, diatomite and perlite (5). Diatomite (also called diatomaceous earth or kieselguhr) is a biogenic siliceous sedimentary rock composed of the fossilized skeletal remains of unicellular organisms, particularly diatoms (6). Due to its specific properties, such as porous structure, high surface area and adsorption capacity, low thermal conductivity and density (7), nearly 99% of the beer production today is filtered through diatomite (8). In order to reach the desired level of clarity and improve the biological stability and colloid stability of beer, all microorganisms and protein particles still in suspension after fermentation are removed by diatomite filter aid (9). During beer filtration process, 1 L of clarified beer consumes an average of 1–2 g of raw diatomite and produces 17.14 g spent diatomite (10). So approximately 86,000 metric tons spent diatomite are discharged annually in China (11), but only small amounts of them are reused in industrial fields such as building materials, pavement materials, environmental protection and chemical industry (9,12–15). Large numbers of spent diatomite are piled on land, which does not only occupy large quantities of land, but also pollutes the environment because it releases carbon monoxide and carbon dioxide to the atmosphere. The regeneration, recycling and disposal of spent diatomite is a major challenge worldwide due to its serious environmental, sanitary and economical effects (16).

For the purpose of environment protection and resource conservation, in recent years, some researches have focused on how to improve the regeneration methods of brewery spent diatomite. Meffert et al. (17) reported a method to regenerate brewery spent diatomite by treatment with aqueous alkaline solution, so as to regain the filter aid activity of spent diatomite. Tsai et al. (18) studied the activation regeneration methods of brewery spent diatomite, including thermal regeneration using a horizontal rotary furnace and chemical regeneration carried out by acid and alkaline agents. Mei and Hui (11) also reported the thermal regenerate method of brewery spent diatomite as an efficient adsorbent. However, thermal regeneration usually has some disadvantages such as high cost, complicated equipment and low efficiency. And chemical regeneration usually brings the secondary pollution to the environment because of the excessive use of acid and alkaline agents (19). Therefore, a more environment-friendly and cost-effective method must be used to regenerate brewery spent diatomite.

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.08.004

Brewery spent diatomite contains large quantities of readily biodegradable organic nitrogen (mainly proteins), which is conducive to breeding of ammonifying bacteria. And it also contains a variety of microorganisms, which mainly come from the beer fermentation process and raw materials needed for beer production (20). So, brewery spent diatomite can be used as a bacteria source of ammonifying bacteria. Considering these factors, biological regeneration method may be an effective and convenient method to regenerate brewery spent diatomite. However, little information presents the biological method for regenerating brewery spent diatomite.

In this study, an ammonifying bacteria was screened out from brewery spent diatomite with a high protein and nitrogen content. The strain was identified by morphological, physiological, biochemical methods and 16S rDNA sequence. Then the protein degradation characteristics of the strain were further analyzed. In addition, the feasibility of using this strain for deproteinization of brewery spent diatomite was evaluated. This study aimed to provide a new method for biological regeneration of high-protein brewery spent diatomite.

MATERIALS AND METHODS

Materials and media Brewery spent diatomite in this study was provided by Tsingtao Brewery (Qingdao, Shandong Province, China), produced in the beer filtration process. Rice protein was purchased from Wuxi Jinnong Biotechnology Co., Ltd. (Wuxi, Jiangsu Province, China). All reagents used in this study were of analytical grade or better.

Enrichment medium was made up of peptone 5 g, K_2HPO_4 0.5 g,$MgSO_4 \cdot 7H_2O$ 0.5 g, and deionized water 1000 mL, pH 7.0.

Isolation medium was prepared with enrichment medium and 2% agar.

Casein medium contained (per liter) 4 g casein, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.36 g KH_2PO_4, 1.070 g $Na_2HPO_4 \cdot 10H_2O$, 0.16 g NaCl, 0.002 g $CaCl_2$, 0.002 g $FeSO_4 \cdot 6H_2O$, 0.014 g $ZnCl_2$, and 20 g agar, adjust pH to 6.5–7.0.

Luria–Bertani (LB) medium consisted of NaCl 10 g, yeast extract 5 g, tryptone 10 g, and deionized water 1000 mL, pH 7.0.

All the above mediums were sterilized at 121°C, 0.1 MPa for 20 min.

Protein-degrading medium was made up of rice protein powder 10 g, glucose 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 2 g, and deionized water 1000 mL, autoclaved at 115°C, 0.1 MPa for 30 min.

Enrichment, isolation and purification of ammonifying bacteria Approximately 10 g of brewery spent diatomite sample was added to 250 mL flask containing 90 mL enrichment medium, shake-flask cultivated in a dark incubator at 30°C, 150 rpm. After culturing for 5 d, the enriched culture broth was transferred to the new enrichment medium at a 10% ratio for further culture, and continuously cultured for 20 d to eliminate other miscellaneous bacteria. In order to select ammonifying bacteria, NH_4^+ –N production was detected by Nessler's reagent. The presence of yellow indicated that the culture broth contained ammonifying bacteria.

After enrichment culture, the culture broth made Nessler's reagent turn darker yellow was selected and diluted into five gradients (10^{-1}–10^{-5}) by using 0.85% (w/v) sterile saline. Diluted solutions (10 μL) taken from 10^{-3}, 10^{-4}, and 10^{-5} dilutions were uniformly coated on plates of casein medium and incubated at 30°C until colonies and casein hydrolytic circles were formed. According to the ratio of hydrolytic circles diameter to colonies diameter (H/C), ammonifying bacteria with proteolytic activity was selected. Then the bacterial colony producing casein hydrolysis circle was streaked onto isolation medium. The isolated colonies were purified by streak culturing three times and saved at 4°C for further identification.

Identification of screened strain by morphological, physiological, biochemical and molecular methods The colony morphology, cell morphology and gram pattern of screened strain were examined. The characteristics of bacterial colony included shape, color, size, transparency and edge. The cellular morphology of the strain was observed by a scanning electron microscope (JSM-840, JEOL Ltd., Tokyo, Japan).

The screened strain was also identified physiologically and biochemically according to Cay and Dong (21).

Moreover, the screened strain was identified by 16S rDNA sequencing. Universal 16S rDNA PCR primers (forward primer Bsf8/20:5′-AGAGTTTGATCCTGGCTCAG-3′; reverse primer 1512R:5′-GGTTACCTTGTTACGACTT-3′) and bacteria colony DNA were used for the amplification of 16S rDNA genes. The PCR program was performed as follows: Denaturation at 95°C for 10 min, 30 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. Amplified PCR products (5 μL) were loaded on a 1% (w/v) agarose gel electrophoresis with 1 μL loading buffer. The amplification products were sent to Harbin Boshi Biotechnology Co., Ltd. (Harbin, Heilongjiang Province, China) for purifying and DNA sequencing. The sequencing result was analyzed and aligned using BLAST (NCBI). 16S rDNA phylogenetic tree was constructed based on neighbor-joining method using MEGA 5.1.

Protease production by the screened strain The screened strain was transferred to LB medium with an inoculum of 5% (v/v), shake-flask cultured at 30°C, 150 rpm for 24 h. Bacterial fermentation broth centrifuged at 8000 rpm for 10 min and collected the fermentation supernatant. The supernatant contained crude enzymes. The protease activity of crude enzymes was measured using Folin method according to Chinese national standard "Proteinase Preparations" (22). In this assay, 1 unit of protease activity was defined as 1 mL enzyme liquid hydrolyzed casein releasing 1 μg tyrosine per min under the standard assay conditions. The crude enzymes were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to verify whether an extracellular protease was secreted by the screened strain.

Optimization of protein degradation by the screened strain The selected strain was inoculated into enrichment medium and incubated at 30°C, 150 rpm to logarithmic growth phase (OD_{600} = 0.8–1.2). Bacterial cells were collected by centrifugation (5000 rpm for 5 min at 4°C), washed twice and diluted to the original volume with 0.85% (w/v) sterile saline to prepare bacterial cell suspension. This cell suspension was inoculated into protein-degrading medium with rice protein as the sole nitrogen source. The optimal carbon source, initial pH value, bacterial inoculum size and culture temperature of protein degradation were determined using one-variable-at-a-time method.

The effect of different carbon sources (glucose, lactose, maltose, sucrose and soluble starch) was studied. Glucose in protein-degrading medium was replaced by the other four various carbon sources. Initial pH value, bacterial inoculum size and culture temperature were set as 7.0, 20% and 35°C, respectively.

The effect of initial pH was studied by adjusting the initial pH value of protein-degrading medium to 5.0, 6.0, 7.0, 8.0, and 9.0. Bacterial inoculum size (20%) and culture temperature (35°C) were constant.

Bacterial cell suspension was inoculated into protein-degrading medium at pH 7.0 with various inoculums of 1%, 5%, 10%, 15%, 20%, and 25% and cultured at 35°C to study the impact of bacterial inoculum size.

Bacterial cell suspension was inoculated into protein-degrading medium (pH 7.0) with an inoculum of 20% and cultured at 20°C, 25°C, 30°C, 35°C, and 40°C study the effect of culture temperature on protein degradation.

All experiments were performed in a shake flask for 48 h at 150 rpm, and the remaining protein concentration was determined. Proteolytic activity of the strain was represented by protein degradation rate in 48 h.

Degradation of protein in optimal conditions According to the above mentioned optimal conditions for protein degradation and experiment method, the selected strain was inoculated into protein-degrading medium and shake-flask cultured in optimal conditions. And the flasks without bacterium functioned as controls. The remaining protein concentration in the medium supernatant was determined at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, 40, and 48 h. Concentration of producing NH_4^+–N in the supernatant was determined every 4 h. Types and contents of free amino acids (FAA) present in the supernatant were also analyzed. A high proteolytic activity *Bacillus subtilis*, purchased from Guangdong Huankai Microbial Technology Co., Ltd. (Guangzhou, Guangdong Province, China), was used to contrast with the selected strain. The protein concentration was determined by the method of Bradford (23) using bovine serum albumin as the standard. NH_4^+–N concentration was determined via Ammonia gas-sensing electrode method (24). Types and content of free amino acids was analyzed by Aglient 1260 RP-HPLC. The chromatographic separation was carried out on SHISEIDO C18 column (4.6 × 250 mm, 5 μm) with a column temperature of 40°C and a flow rate of 1.0 mL/min. The detection wavelength was 254 nm. Mobile phase consisted of solvent A: 0.1 M sodium acetate solution (pH 6.5), acetonitrile (97:3) and solvent B: acetonitrile/water (8:2). Identification and quantification of free amino acids were carried out by comparison with an amino acids mixture standard solution (Sigma Chemical, San Francisco, CA, USA).

Biodegradation of protein in brewery spent diatomite at laboratory scale In order to explore a new biological method for regeneration of high-protein brewery spent diatomite, the selected strain was applied for deproteinization of spent diatomite. 5 mL cell suspension of the selected strain with a turbidity of 1.0 at OD_{600} (contained approximately 1×10^{10} cells) was inoculated into 100 g freeze-dried spent diatomite sample. Another set without bacterium was kept as a control. The moisture of spent diatomite in each set was maintained at 50% throughout the experiment by sprinkling water periodically when required. Each set was incubated at 30°C in the dark for 14 d. Diatomite samples were collected every day from each set and the concentrations of protein were analyzed. Experiments were performed in triplicate.

Proteins of brewery spent diatomite were mainly derived from the beer-brewing raw materials (barley malt and rice). The extraction of proteins from spent diatomite used alkaline method, which had been widely used for protein extraction from plant sources (25). The proteins of spent diatomite were extracted with 0.01M NaOH at pH

12, 70°C and solvent/flour ratio of 40:1 for 1 h. Protein concentration of the extracting supernatant was determined by the method of Bradford.

After deproteinization, the specific surface area of spent diatomite and bio-regenerated diatomite were determined at −196°C according to Brunauer, Emmett and Teller (BET) gas adsorption method using ASAP 2020 surface area analyzer (Micromeritics Co., Norcross, GA, USA).

Statistical analysis All data presented in this paper were averages of three independent tests. Data were expressed as the mean and standard deviation (mean ± SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by *t*-test (IBM SPSS Statistics 20.0). The results were considered to be statistically significant for P value <0.05.

RESULTS AND DISCUSSION

Screening of the ammonifying bacteria with proteolytic activity In this study, a strain, designated BSD1, was isolated from spent diatomite of Tsingtao Brewery. As shown in Fig. S1, the H/C values of the strain BSD1 was higher than 3.0, indicating strain BSD1 had proteinase activity. The bacterial culture broth of BSD1 can make Nessler's reagent turn orange, illustrating strain BSD1 was an ammonifying bacteria.

Identification of the ammonifying bacteria As shown in Fig. S2, the bacterial colonies of BSD1 on isolation medium were white, round and opaque with diameters from 1.0 to 3.0 mm. The surface was slightly wet and raised, and the edge was smooth. Strain BSD1 was positive in Gram-staining. As shown in Fig. 1, under the scanning electron microscope, the bacterial cells of BSD1 were rod-shaped, with a width of 0.7 μm and a length of 1.5−5.0 μm, and had flagella indicating strain BSD1 was motile.

The results of biophysical and biochemical identification of the strain BSD1 were shown in Table 1. Strain BSD1 could tolerate 7 % (w/v) NaCl, Temperature range for growth was from 20°C to 50°C. The pH range for growth was between 6.0 and 9.0. The methyl red and Voges−Proskauer test showed BSD1 could break down glucose to produce pyruvate but could not produce acetyl methyl carbinol. Strain BSD1 was tryptophanase, phenylalanine deaminase negative and catalase, amylase, urease, caseinase, gelatinase positive. And it could ferment glucose, lactose and glycerin to produce acid, but neither of sucrose, maltose and D-xylose. Physiological and biochemical characteristics of the strain BSD1 were similar with several strains having been previously identified as the genus *Lysinibacillus* (26−28). So, the strain BSD1 was preliminarily identified as *Lysinibacillus*.

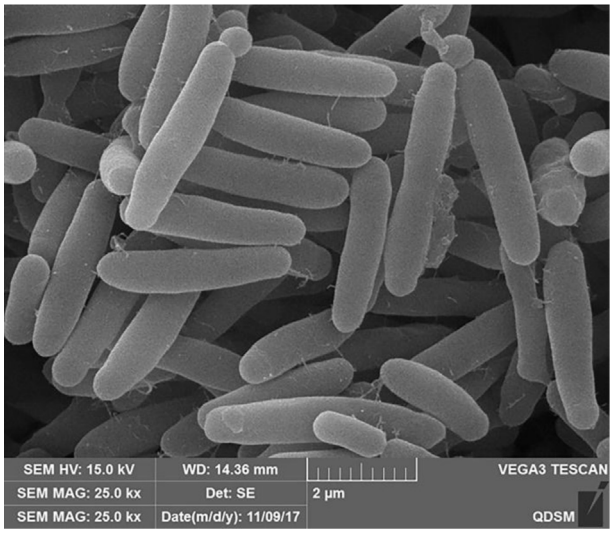

FIG. 1. The cell morphology of strain BSD1 under scanning electron microscope.

The 16S rDNA sequence of the strain BSD1 was submitted to GenBank (NCBI) and got the accession number MG770404. The comparison between 16S rDNA sequence of BSD1 and NCBI database showed that the homology between BSD1 and *L. fusiformis* was 99%. The phylogenetic trees based on 16S rDNA sequence of the strain BSD1 are shown in Fig. 2. According to the result and analysis of colony morphology, cell morphology, physiological and biochemical characteristics and 16S rDNA sequence, the strain BSD1 was identified as *L. fusiformis*.

Production of crude protease To explore protease production of strain BSD1, the protease activity and SDS-PAGE of crude enzymes were evaluated. The protease activity of crude enzymes in LB medium was as high as 33.4 U/mL. In SDS-PAGE the crude enzymes displayed several bands with a molecular mass of approximately 63 kDa, while the same bands were not found in LB medium without strain BSD1 (Fig. S3). These results confirmed that the strain BSD1 could secrete extracellular protease for protein degradation. Some *bacillus* species have been previously shown to produce extracellular proteases for deproteinization. Mechri et al. (29) reported that a new extracellular 51 kDa-protease was secreted from *L. fusiformis* C250R and it could be used as an effective cleaner in detergent industry to remove proteinaceous stains. Prabha et al. (30) demonstrated that *L. fusiformis* AU01 could produce an extracellular 48 kDa-protease, which belonged to the family of serine metalloproteases. Suppiah et al. (31) confirmed that *L. fusiformis* could secrete an extracellular protease with a molecular mass of 29 kDa. Additionally, Srinivasan et al. (32) reported the production of a thermostable extracellular protease of molecular mass of 68 kDa from *Bacillus* sp.

Optimal deproteinization In order to enhance protein degradation ability of the strain BSD1 and reach maximum protein degradation level, the effects of initial pH value, carbon source, culture temperature and bacterial inoculum size on proteolytic activity of strain BSD1 were investigated. The results are shown in Fig. 3. The strain BSD1 presented higher proteolytic activity over a range of pH 6−8. And the highest protein degradation rate (62%) was observed at pH 7 (Fig. 3A). This illustrated strain BSD1 could secrete neutral proteinases which kept maximum activity at pH 7. A neutral initial pH had been previously reported to be optimum for protease production by some microbes in *Bacillacease* such as *L. fusiformis* F3G5, *Bacillus amyloliquefaciens* and *Bacillus subtilis* (33−35). As shown in Fig. 3B, among the different carbon sources, protein degradation rate reached maximum in glucose (64%), followed by maltose (54%) and starch (47%), similar to the results of Mechri et al. (29). This indicated that different carbon sources had a various influence on the proteolytic activity. For strain BSD1 glucose was the best carbon source. It was probably due to most microorganisms preferring to utilize glucose as a carbon source (36). Similar results had been revealed by some earlier researches (37,38). Fig. 3C shows that the optimum temperature for protein degradation was 30°C and the degradation rate reached up to 74%. This result confirmed that the protease produced by the strain BSD1 might exhibit highest activity at 30°C. It was previously reported that 30°C was an optimum temperature for protease production by *Bacillus* sp. MIG, *Bacillus licheniformis* and *B. coagulans* (39,40). As shown in Fig. 3D, the protein degradation rate was 85% at bacterial inoculum size of 5%, significantly more ($P< 0.05$) than that at the inoculum of 20%. It might be attributed to that small inoculum size had higher surface area to volume ratio, which resulted in the increased production of protease. And high inoculum sizes might cause the lack of oxygen and increase competition towards nutrient. However, if the inoculum size was too small, insufficient number of bacteria would lead to a reduced amount of protein

TABLE 1. Physiological and biochemical characteristics of the strain BSD1 and several strains of the genus *Lysinibacillus*.

Characteristic	BSD1	*Lysinibacillus fusiformis*	*Lysinibacillus Sphaericus*	*Lysinibacillus xylanilyticus*
Cell length (μm)	1.5–5.0	1.5–5.0	1.5–5.0	3.0–5.0
Gram stain	+	+	+	+
Motility	+	+	+	+
Temperature range for growth (°C)	20–50	17–40	10–40	10–37
pH range for growth	6.0–9.0	6.0–9.0	6.0–9.0	5.0–9.0
Growth without NaCl	+	+	+	+
Growth in:				
2 % (w/v) NaCl	+	+	+	+
5 % (w/v) NaCl	+	+	+	+
7 % (w/v) NaCl	+	+	–	–
10 % (w/v) NaCl	–	–	–	–
Methyl red test	+	ND	ND	ND
Voges–Proskauer test	–	–	–	–
Catalase test	+	+	+	+
Indole test	–	–	–	–
Phenylalanine deaminase test	–	ND	ND	ND
Hydrolysis of:				
Starch	+	ND	ND	ND
Urea	+	+	–	–
Casein	+	+	+	+
Gelatin	+	+	+	+
Acid production from:				
Sucrose	–	v	–	–
Lactose	+	ND	ND	ND
Glucose	+	+	–	–
Maltose	–	–	–	–
D-Xylose	–	–	–	–
Glycerin	+	ND	ND	ND

+, positive result; –,: negative result; v, variable; ND, no data available.

degradation (41,42). So, 5% was an optimum inoculum size for protein degradation. The result was in accordance with earlier study by Abusham et al. (35). Thus, inoculation with 5% of bacterial inoculum size, in a medium with glucose as carbon source, initial pH value of 7.0 and incubated at 30°C, were the optimum conditions which resulted in the maximum protein degradation activity of the strain BSD1. After optimization, the strain BSD1 exhibited a protease activity of 6.12 U/mL in rice protein medium. Previous studies had reported the application of protease-producing bacterium for deproteinization. Wang et al. (43) used *Bacillus* sp. TKU004 for deproteinization of squid pen and both the production of protease and the resulted protein removal of squid pen attained the optimum after optimization. They were 0.065 U/mL and 73%. Wang and Chio (44) reported the utilization of *Pseudomonas aeruginosa* K-187 for deproteinization of shrimp and crab shell waste and the protease activity and deproteinization rate were 0.17 U/mL and 47%, respectively, after 5 days fermentation.

Protein degradation ability of BSD1 To compare the protein degradation activity of strain BSD1 with a well-known high proteolytic activity *Bacillus subtilis* (45) and explore the time-courses of protein degradation, protein degradation curves of the strain BSD1 and *Bacillus subtilis* were investigated. As shown in Fig. 4A, the remaining protein concentration in medium supernatant adding BSD1 decreased dramatically at the first 8 h. After 8 h, the speed of protein degradation slowed down. After degrading for 20 h, the protein concentration decreased from 156.8 mg/L to 19.2 mg/L, and the degradation rate was up to 88%, while the degradation rate of *Bacillus subtilis* was only 75% at this time. Compared with *B. subtilis*, the strain BSD1 exhibited higher protein degradation capacity in optimal conditions. The result confirmed that the maximum protein degradation ability of strain BSD1 could surpass some common microorganisms which had ever been used for deproteinization. The increase of protein concentration after 32 h might be caused by most bacterial cells of strain BSD1 died when the nutrient of culture media was exhausted and the intracellular proteins were released to the medium supernatant.

Fig. 5 shows types and contents of FAA generated in the medium supernatant after adding the strain BSD1. Total FAA content of the flasks with strain BSD1 increased from 27.0 mg/L to 50.9 mg/L in 48 h. Total FAA content almost remained unchanged at the first 4 h. Corresponding to the change of FAA, protein concentration decreased rapidly in 0–4 h (Fig. 4A). The result confirmed that proteolytic enzymes secreted by BSD1 were mainly

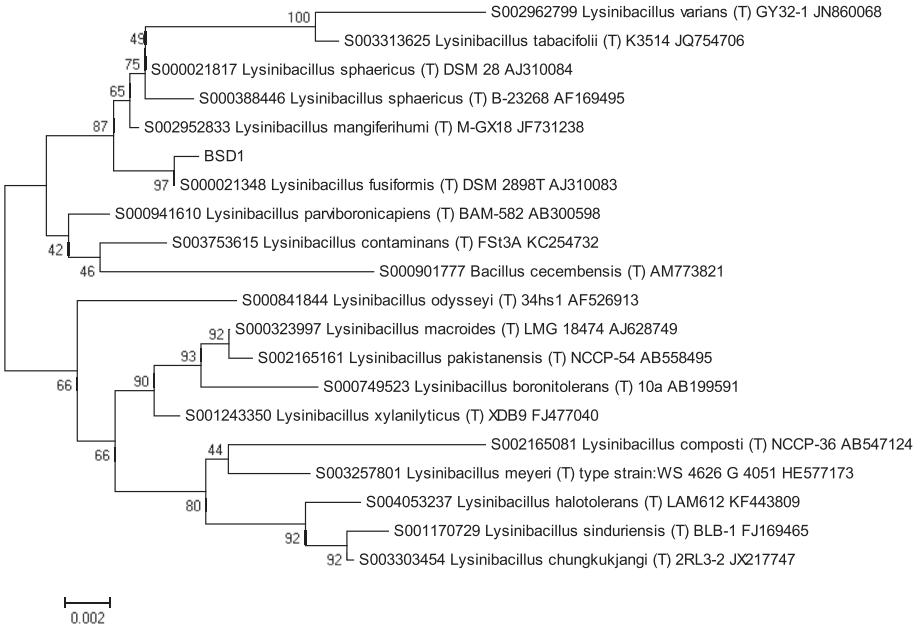

FIG. 2. The phylogenetic trees based on 16S rDNA sequence of the strain BSD1.

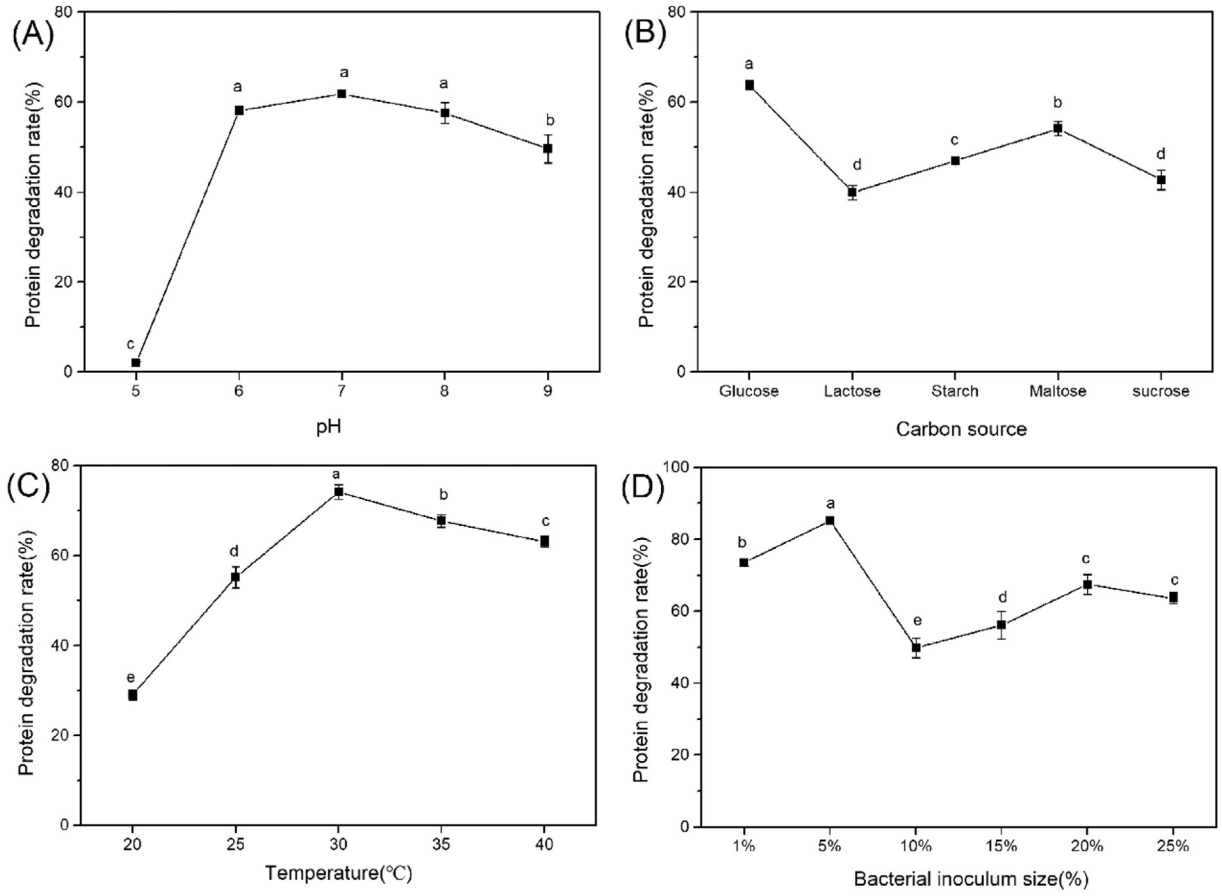

FIG. 3. The effect of initial pH value (A), carbon source (B), culture temperature (C) and bacterial inoculum size (D) on protein degradation by the strain BSD1. The different letters represent significant differences ($P < 0.05$).

endopeptidases in this process. The role of endopeptidases was to cleave a large molecular weight polypeptide chains from the inner regions and formed small peptides (46). Total FAA content rose rapidly within 4–8 h (mainly phenylalanine, tyrosine, proline, alanine increased). After 8 h, the content of lysine, threonine and histidine slightly increased. The increase of FAA indicated that exopeptidases were secreted by BSD1. Exopeptidases acted only near the ends of peptide chains and they could hydrolyze peptides into free amino acids at the free carboxy terminus or the free amino terminus of peptide chain (46). Among the generating 17 kinds of FAA, only seven kinds of FAA (Phe, Tyr, Pro, Ala, Lys, Thr, and His) increased but the other kinds of FAA were almost unchanged. This illustrated that proteolytic enzymes secreted by BSD1 had a strong hydrolysis preference for the chemical bonds composed of these seven kinds of FAA.

NH_4^+–N generation in protein degradation process is shown in Fig. 4B. NH_4^+–N concentration in the medium supernatant was extremely low in the first 8 h and then increased slowly after 8 h. NH_4^+–N concentration increased rapidly during 32–48 h and reached 232.2 mg/L at 48 h. The increase of NH_4^+–N concentration after 8 h might be attributed to the decrease of the content of two kinds of FAA (Pro and Ala). This demonstrated that BSD1 might secrete some amino acid deaminases and convert FAA to ammonium or ammonia. Previous studies had reported some microorganisms exhibited ammonifying capacity. Niewiadomska et al. (47) reported that some bacteria, actinomycetes and fungi in the soil could utilize proteins and amino acids as nutritive and energetic substrates. Spano et al. (48) revealed that *Lactobacillus* strains isolated from a typical red wine could be used for the deamination of arginine.

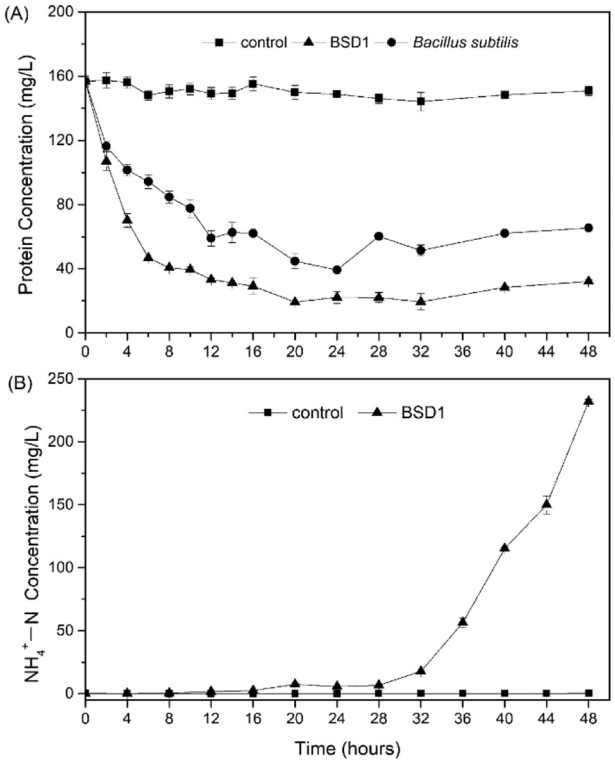

FIG. 4. Protein degradation by the strain BSD1 and *Bacillus subtilis* (A); NH_4^+–N generation in protein degradation process (B).

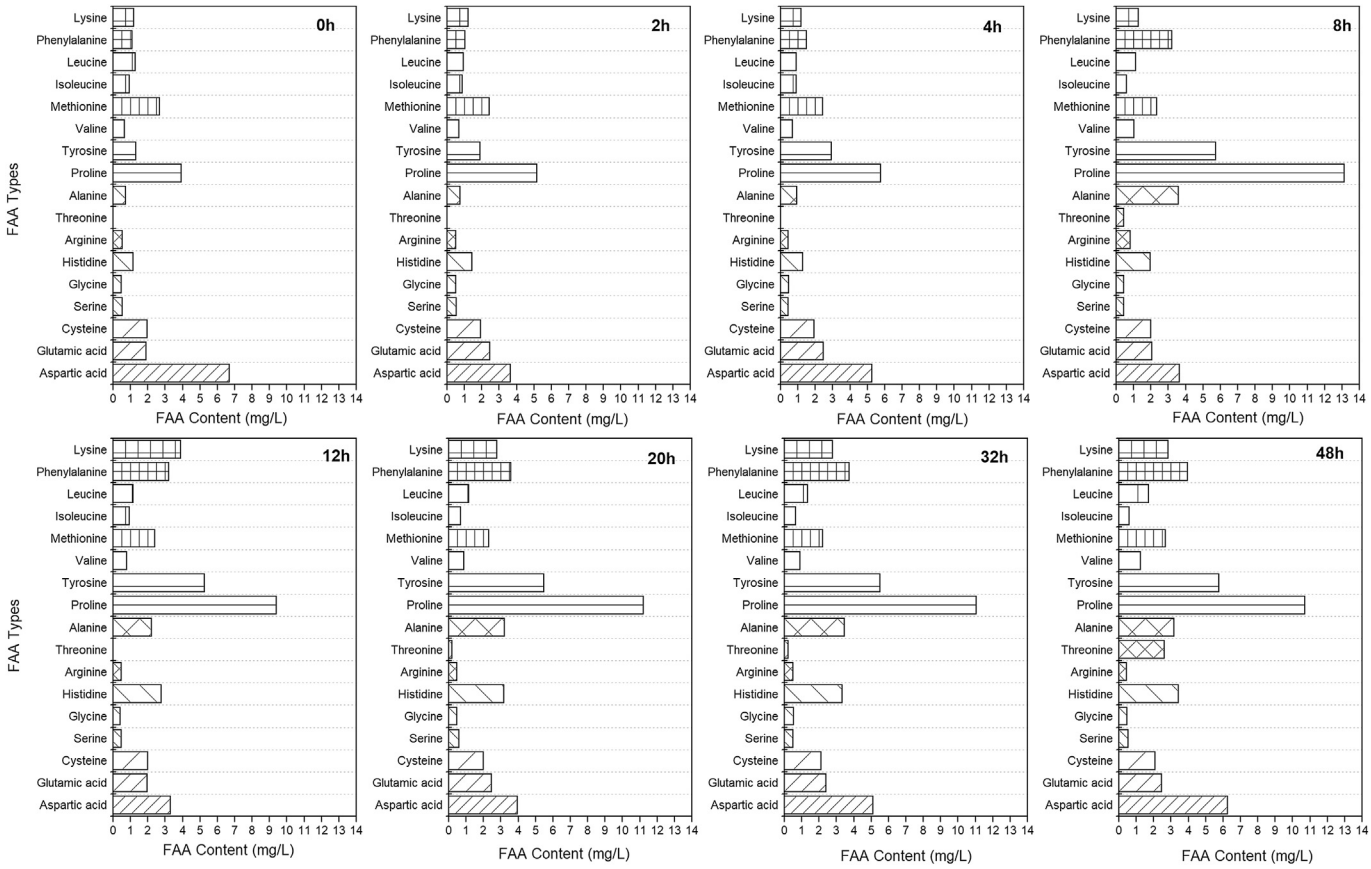

FIG. 5. Types and contents of FAA generating in the medium supernatant.

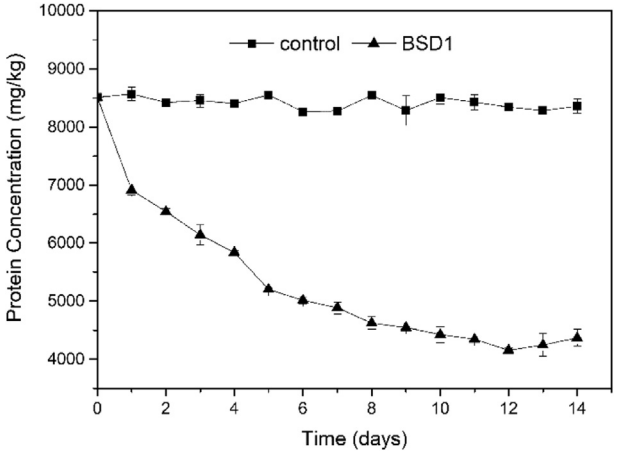

FIG. 6. Biological deproteinization of brewery spent diatomite by the strain BSD1.

Deproteinization of brewery spent diatomite with the strain BSD1 In order to explore a new method for bio-regeneration of brewery spent diatomite, biodegradation of protein in spent diatomite by the strain BSD1 was evaluated. As shown in Fig. 6, when strain BSD1 was inoculated to spent diatomite, the protein concentration decreased dramatically at the first 5 days. After 5 days, microbial activity of strain BSD1 decreased and the degradation speed of protein became slower. After deproteinization for 12 days, the protein concentration of spent diatomite decreased from 8513 mg/kg to 4155 mg/kg and 51% of proteins in spent diatomite were degraded. This result indicated that strain BSD1 could be used for biological deproteinization of brewery spent diatomite. After deproteinization, the textural parameters of spent diatomite (SD) and bio-regenerated diatomite (BRD) samples such as the specific surface area, total pore volume and average pore size were analyzed. As shown in Table 2, after biological deproteinization the specific surface area of diatomite increased from 16.10 to 26.89 m^2/g and total pore volume increased from 0.064 to 0.116 cm^3/g. And the average pore size was decreased, illustrating that the micropore in diatomite increased due to macromolecular proteins were degraded into small molecules by strain BSD1 and more micro pores were released. Compared with the traditional thermal regeneration method in previous studies (18,49), the regenerated diatomite was superior in pore structures after biological regeneration treatment. Thus, the application of strain BSD1 for regeneration of high-protein brewery spent diatomite was feasible.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.08.004.

TABLE 2. Textural parameters of spent diatomite and bio-regenerated diatomite.

Sample	Specific surface area S_{BET} (m^2/g)	Total pore volume (cm^3/g)	Average pore size (nm)
SD	16.10	0.064	9.42
BRD	26.89	0.116	8.83

ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of China (No. 51579228). Appreciation is expressed to Tsingtao

Brewery, Shandong, China for providing the spent diatomite samples.

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