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The Growth, Effect of COD-Reduction, and Flocculation Characteristics of *Candida rugosa* in Sugar Beet Stillages

LEE, KI-YOUNG*

Department of Food and Nutrition, Hoseo University
29-1 Sechul-Ri, Asan-Kun, Chungnam 337-850, Korea

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Yeast fermentation at 40°C was conducted for microbial protein production and COD reduction in three different sugar beet stillages by a thermo- and acid-tolerant yeast *Candida rugosa* isolated from East Africa. The assimilation proceedings of some main components such as protein, carbohydrate, total titrable acids and glycerol in stillages were observed with growth kinetics of the yeast. Most of glycerol and organic acids were rapidly assimilated at the beginning of the fermentation. Protein assimilation was slowly accelerated with the proceeding of fermentation time and its assimilation rate reached only 14.2%-28.4%. Though *Candida rugosa* was a flocculent yeast, the flocculation characteristics of the yeasts grown in three stillages were different from each other.

Stillage, also termed as distillery alcohol slop, or spent wash, is a distillery waste water which is characterized by a mostly soluble and biodegradable solid content with high ash content, low pH, and high temperature. It posseses large amounts of potassium, sodium, sulphate and heavy degradable compounds such as melanoidine, gums and protein, and betain in case of beet stillage, and a COD (Chemical Oxygen Demand) value between 15 and 176 g per liter (19). It is generated from sugar cane and beet juices, or their molasses, and other sugar containing mashes of grains, potatoes, and maize. Increased worldwide attention is being given to ethanol production from agricultural materials through biological conversion due to the fossil energy shortage. Especially in Brazil, the National Alcohol Program was established in 1875 (15), and the production of ethanol was increased by more than 20 times greater during a span of 15 years. This posed a serious threat to water quality. In a typical ethanol distillery, 10 to 15 liters of stillage, which have about 40 to 80 g per liter of COD, are given out for every liter of ethanol produced. The bioconversion of agro-industrial wastes into feed and fuel products is an interesting field of technical, economical treatment of accumulating pollution-inducible wastes in the aspects of raw material supply and renewable energy sources. The feed yeast production from stillage has been long investigated and industrially applied in a number of countries such as Hungary, the Soviet Union and Taiwan (19). For the economical process developement of SCP (Single Cell Protein) production, some properties of strains play significant roles. Regarding such properties, the important parameters for the yeast biomass production from agricultural effluents are presented in Table 1.

Higher specific growth rate and biomass yield of yeast are meaningful properties of yeast in SCP production; however, as an important practical means of SCP production considering the treatment cost involved, the avoidance of sterilization, low cooling water consumption and ease of biomass recovery are necessary. For these purposes, such properties of yeast as acid tolerance, thermotolerance, and good flocculation and sedimentation properties are needed. As a stillage treatment in this experiment, an aerobic yeast fermentation was investigated and an acid thermotolerant yeast, *Candida rugosa*, was selected, which was isolated in Sudan of East Africa. This yeast showed a high biomass yield and high specific growth rate at 40℃ (3, 11). Kinetic behavior and flocculation characteristics of *C. rugosa* at 20℃, 30℃, and 40℃ were also studied (12). In the present study, the growth

*Corresponding author
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Table 1. Important process conditions and corresponding properties of the strains for the feed yeasts production from agricultural effluents

Aim	Process conditions and corresponding properties of yeast strain
No sterilization	−Short residence time and high yeast density in fermenter or fermentation at lower pH
	−Flocculation characteristics of the yeast for recycling
	−Acid tolerance of the yeast
Low cooling water consumption	−Fermentation at higher temperature
	−Thermotolerance of the yeast
High productivity	−High yeast density in fermenter
	−High specific growth rate of the yeast
	−High cell yield from the effective utilization of broad carbon compounds spectrum
Easy recovery	−Flocculation characteristics of the yeast

of *C. rugosa*, corresponding COD reduction and substrate assimilation in three different beet stillages are reported.

MATERIALS AND METHODS

Microorganism

The yeast *C. rugosa* isolated from Sudan was identified by the method of Barnett (2). The strain was maintained on the stillage slant agar at 6℃ and reinocculated every 6 months.

Growth Medium

Three stillages, with different total solid content (A: 60), B: 83.6, C: 103.7 g/l) on different working days, were selectively obtained from the distillery of Versuchs- und Lehranstalt fuer Spiritusfablikation and Fermentationstechnologie, Berlin. Analysis results showed that the composition of Sample B was similar to that of the annual average range and Sample B contained more water. In contrast, Sample C comprised not only non-fermented fructose, but also a relatively large amount of volatile fatty acids and sulfite which could inhibit the metabolism of yeast (3). Therefore, Sample C was concentrated to 65% total solid content and, when necessary, diluted with distilled water to ferment it again. In this experiment, Sample C was prepared to contain 8% of total solids and was centrifuged to get rid of sedimented solids containing dead yeasts and other insoluble suspended materials.

Fermentation Conditions

Batch culture experiments were conducted in a 1 litre magnetic stirred fermenter fitted with a dissolved oxygen analyzer and automatic pH and temperature controller, which was assembled in our laboratory. Working volume was 850 m*l* and temperature was maintained at 40℃. Agitation speed was 600 rpm with an aeration rate of 1.8 vvm. Dissoved oxygen, measured with a galvanic electrode, was regulated at over 20% of the saturation under these conditions. 50 m*l* of one day shaking cultured stillage was used as an inocculum and all experiments were carried out under aseptic conditions.

Analytical Methods

Biomass was measured by dry weight method after centrifugation (10 min at 5000 g). They were washed twice with distilled water and weighed after 24 h period at 105℃. COD was measured by the standard method of DIN 38409 (7) with a thermoreactor TR 105 (Merck, Darmstadt). Protein concentration was determined by the bieuret method as described by Herbert *et al.* (10) using bovine serum albumin as a standard and crude protein by Kijeldahl method. Total acidity was measured by titration against 0.01 M NaOH using bromothymol blue as an indicator and expressed as lactic acid equivalents. Total carbohydrate was determined by the anthrone method (20) and glycerol by the enzymatic method of Boeringer Mannhein (6). The extracellular protease activity was measured as follows: in a reagent glass, 0.5 m*l* of 0.05% casein yellow solution and 0.5 m*l* incubation buffer were mixed at 37℃ and incubated for 20 min. Then, 4.8 m*l* of 0.5 M perchloric acid was added and incubated again for 10 min. After that, the incubated sample was centrifuged to settle not-reacted casein yellow and 1 m*l* supernatant was removed and was mixed with 1.5 m*l* analysis buffer. The absorbance of this solution was measured at 422 nm. Used reagents were as follows: incubation buffer (0.85% NaCl in 0.1 M NaOH), analysis buffer (standard 0.1 M acetate buffer, pH 4.4).

As a standard protease, neutral protease from *Bacillus polymixa* (Boeringer Mannheim) was used. Flocculation characteristics were tested through the combination of observing sedimentation and the difference in cell concentration. From the stationary phase of fermentation, 25 m*l* yeast culture was removed from the media and washed twice with distilled water. Settling velocities of the cell suspension were determined in a graduated cylinder (volume 25 m*l*: 15 cm long and 1.5 cm i.d.) by measuring interphase height versus time. At the same time, a 50 μ*l* yeast suspension from the 20 m*l*-marking line of the cylinder was taken, diluted 50 fold with distilled water, and the absorbance was measured spectrophotometrically at $\lambda=750$ nm. By comparing this value, measured each time with the value of the beginning, the decrease of yeast concentration in the upper half-phase of yeast suspension during experimental periods can be observed.

RESULTS AND DISCUSSION

Yeast Growth and Assimilation

The growth of *C. rugosa* in the batch fermentation of stillages A, B and C is shown with the assimilation courses of important assimilable compounds such as protein, carbohydrate, total titrable acids, and glycerol in the Fig. 1, 2, and 3.

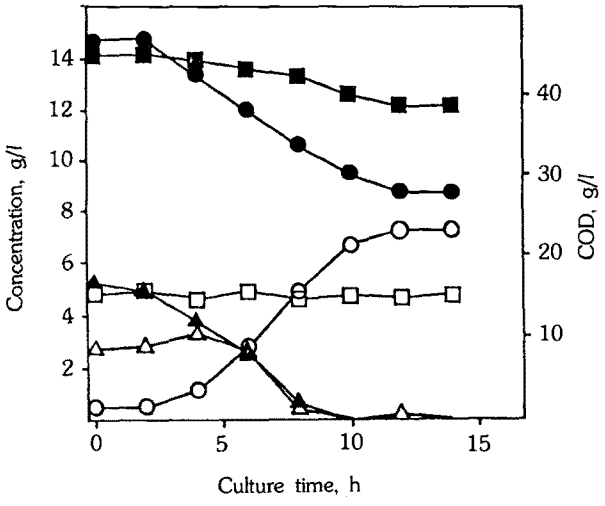

Fig. 1. Batch fermentation kinetics of *C. rugosa*, COD reduction, and consumption of some main compounds in sugar beet stillage A at 40°C.
○ Biomass, ● COD, ■ Protein, △ Glycerol, □ Carbohydrate, ▲ Titrable acids as lactate.

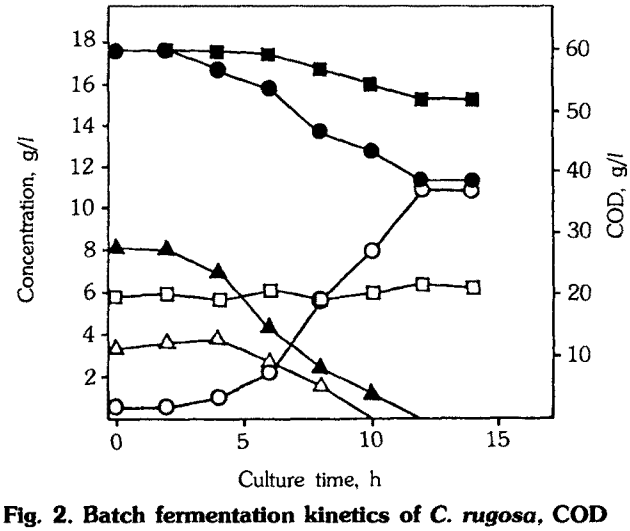

Fig. 2. Batch fermentation kinetics of *C. rugosa*, COD reduction and consumption of some main compounds in sugar beet stillage B at 40°C.
○ Biomass, ● COD, ■ Protein, △ Glycerol, □ Carbohydrate, ▲ Titrable acids as lactate.

With the increase of dry matter in the stillages, the overall growth rate (μ_o) of *C. rugosa* became higher, and the μ_o of the stillage C was especially high. The μ_o values of stillages A, B and C were, respectively, 0.14, 0.16 and 0.19 h^{-1}. Productivity of the biomass and COD reduction rates in the stillages were, in the same turn, 0.70, 0.90 and 1.13 g/l·h and 35.6, 35.5 and 44.3%. In these fermentations, glycerol and organic acids were, relatively speaking, rapidly assimilated at the beginning of the fermentation. Protein assimilation was slowly accelerated with the proceeding of fermentation time. Fig. 4 shows the protease activity of stillage broth and the crude protein content of yeast cells during the whole fermentation period.

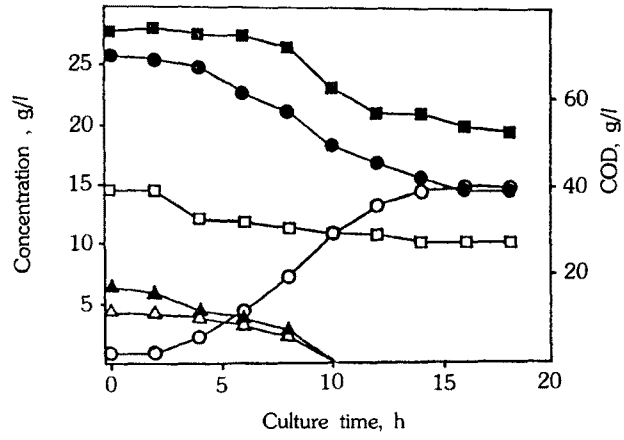

Fig. 3. Batch fermentation kinetics of *C. rugosa*, COD reduction and consumption of some main compounds in sugar beet stillage C at 40°C.
○ Biomass, ● COD, ■ Protein, △ Glycerol, □ Carbohydrate, ▲ Titrable acids as lactate.

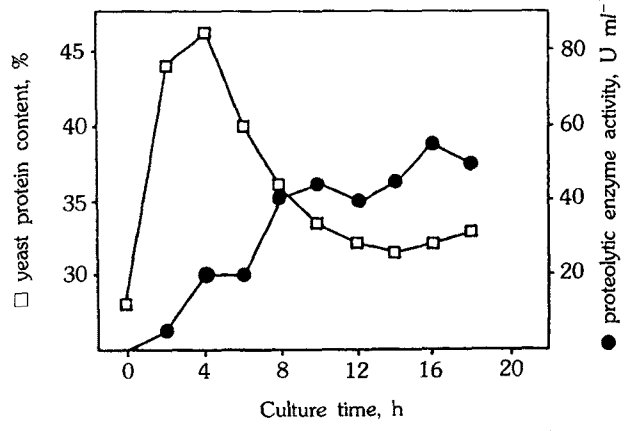

Fig. 4. Dependence of yeast protein content and proteolytic enzyme activity in the fermentation of sugar beet stillage C from the fermentation period.

As fermentation time proceeded, protease activity was increased and, at the end of the fermentation, reached the highest value. These results revealed the protein assimilation somewhat clearly. The low protease activity in stillage C at the beginning was rapidly accelerated after 6 h and slowly increased to the end of the fermentation. In this relation, it seemed that the assimilation of amino acids or added urea in the stillage was prefered over that of protein, due to the low activity of protease at the beginning of the fermentation. The crude protein content of the cell mass was strongly increased at the lag phase of the fermentation to 47%, and decreased with the exponential cell growth. Nevertheless, at the end of the fermentation it increased again slowly in accordance with the assimilation of relatively more proteins. The glycerol concentration in stillage A and B increased slightly within the first 4 h at the beginning of the fermentation, and then decreased. The increase of glycerol at the beginning was probably due to degradation of macromolecular components of the stillages (5).

In the case of stillages A and B, the assimilation of carbohydrates was not revealed clearly. As was already reported (3), stillage A and B did not contain any D-glucose and D-fructose. However, in case of stillage C, the most fermentable sugars were assimilated within the first 4 h of the fermenation. Stillage C comprised some unfermented D-fructose. As was also reported by Quinn and Marchant (16), these sugars could be rapidly assimilated. The glucose and fructose transport is the carrier bound light diffusion, which follows the Michaelis-Menten-Kinetics. Therefore, μ_o of *C. rugosa* in stillage C, which contained D-fructose, could be higher than in the others. In all stillages, organic acids and glycerol were almost all quickly assimilated, but protein assimilation reached only 14.8% in stillage A, 14.2% in B and 28.4% in C. Stillage C contained more assimilable proteins seem to orginate from the cell autolysis (3). Crude protein contents of the produced yeast biomass after fermentation were 35.6% for stillage A, 36.6% for B and 32.8% for C. Biomass yields by the reduced COD value ($Y_{X/COD}$) were 0.43 for stillage A and B, and 0.48 for C. For stillage C, biomass yield was higher, while crude protein content was lower than those of the other two samples.

Flocculation Characteristics

Flocculation characteristics of *C. rugosa* grown in 3 stillages varied from each other as shown in Figs. 5 and 6.

Maximum sedimentation velocity in stillages B and C were 0.35 and 0.24 mm/sec respectively. In these cases, the phase separation between yeast flocks and supernatant were distinct and the difference of two results was seemed to be caused only by the different yeast concentrations. The bright part of the sedimented yeasts

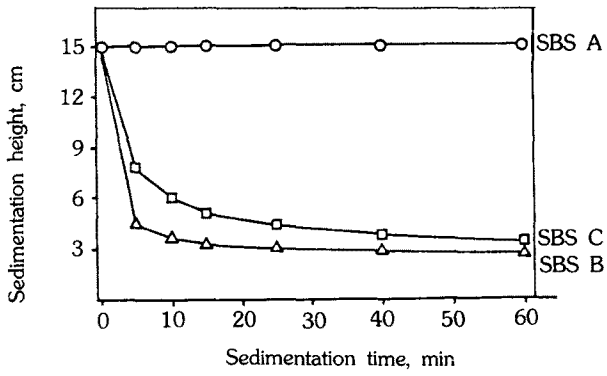

Fig. 5. Dependence of flocculation characteristics of *C. rugosa* grown at 40°C from the Sugar Beet Stillage (SBS) medium.

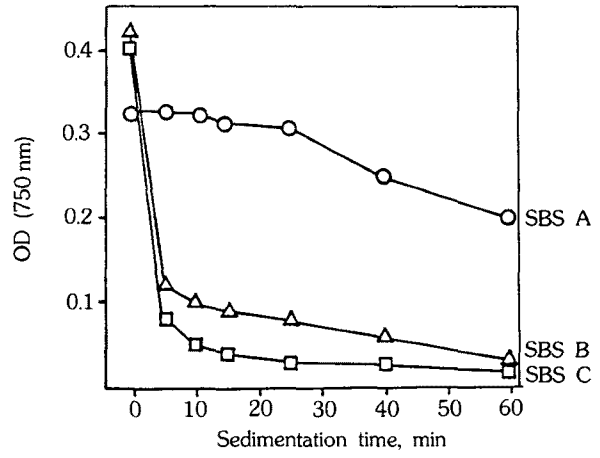

Fig. 6. Dependence of optical density in the upper half of the yeast suspension with the sedimentation proceedings from the Sugar Beet Stillage (SBS) medium.

in stillage B was higher than that in stillage C. On the other hand, the yeasts in stillage A did not form any aggregates after 60 min and sedimented very slowly. After 12 h, the sedimented yeasts were far brighter than those in the stillage B or C. Flock formation of yeast has been shown to be dependant on variable factors such as genetic natures of the yeast (18, 21), fermentation conditions with temperature effect, substrate and bivalent ion concentration (8, 9, 14, 17). Though the causes of different flocculation characteristics of *C. rugosa* in each stillage were not clear, presumably the differences in stillage composition played a significant role in flock formation because other fermentation conditions were constant. As reported by Bajpai and Margaritis (4), stillage contains variable high molecular components such as melanoidines, proteins, polysaccharides and furfurals, which can influence the flocculation characteristics of the yeast. Miki *et al.* (13) and Amri *et al.* (1) have

reported that calcium ions had a special effect on the surface structures of cell wall and thus produced a positive effect on flock formation. According to their reports, 50 mM of $CaCl_2$ solution was added to yeast suspension of stillage A. This caused a sudden flocculation of yeasts and they sedimented easily.

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