Effect of Sodium Chloride on Bakers’ Yeast Growing in Gelatin

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In recent years, industrial fermentation researchers have shifted their attention from liquid to solid and semisolid culture conditions. We converted liquid cultures to the semisolid mode by adding high levels of gelatin. Previous studies on liquid cultures have revealed the inhibitory activity of mineral salts, such as NaCl, on the fermentation of sugars by yeasts. We made a kinetic study of the effects of 1 to 5% (wt/vol) NaCl on the alcoholic fermentations of glucose by Saccharomyces cerevisiae in a growth medium containing 16% gelatin. Our results showed that the effect of high salt content on semisolid culture is essentially the same as the effect on liquid culture; i.e., as the salt content increased, the following occurred: (i) the growth of yeasts decreased, (ii) the lag period of the yeast biomass curve lengthened, (iii) the sugar intake was lowered, (iv) the yield of ethanol was reduced, and (v) the production of glycerol was increased. We observed a new relationship correlating the area of kinetic hysteresis with ethanol production rate, acetaldehyde concentration, and the initial NaCl concentration.

The fermentation of sugars, especially glucose, by Saccharomyces cerevisiae in liquid culture has been one of the most intensively studied phenomena in the history of scientific inquiry. Recently, interest in this area has shifted to solid and semisolid fermentations for two primary reasons: certain products such as fermented foods like miso and soy sauce are becoming more popular worldwide, and stirring and the separation of sugars from natural raw materials are often not required with solid materials; therefore, much energy can be saved by using the natural substrates directly.

We studied kinetic changes at a transition point between the culture in liquid form and in solid form by fabricating a gelatin medium as a semisolid model. A 16% (wt/vol) gelatin concentration was selected as the maximum gelatin level because preliminary studies had shown that a higher concentration (i) made the separation of yeast cells from the gel medium (for measurement of biomass) very difficult, (ii) made pH measurements in the fermenting mash unreliable, and (iii) caused concern about possible aeration of the anaerobic culture after the removal of sampling plugs since the holes made in the gelled medium by the sampling did not close completely, allowing diffusion of ambient air into the openings.

Historical review. The high concentrations of electrolytes in fermented foods and in raw materials such as molasses-enriched sugar cane stalks has motivated studies of the inhibitory effects of high levels of inorganic salts on sugar fermentations of industrial importance. This inhibitory action was first reported by Tajima et al. (12) and Umemoto et al. (18). Tajima et al. (12) have suggested that salt tolerance be added to the list of desirable characteristics of yeast strains used for alcoholic fermentation of molasses. For more convenient experimental methodology, subsequent researchers have replaced the heterogeneous mixture of inorganic salts found in molasses with pure sodium chloride.

To date, however, few kinetic studies have been reported on the effects of sodium chloride on entrapped yeasts growing in semisolid media (16), mainly because it is difficult to track cells attached to solids. Gelatin medium provides an easy way to measure the mass of cells by simply melting the gelatin at 40 to 45°C. The still viable cells are then easily separated from the gel, and the cell-free liquid is handled as a conventional liquid medium.

Summary of previously reported salt effects. Elevated levels of inorganic electrolytes in an otherwise satisfactory liquid growth medium have been found to influence several parameters of yeast activity. (i) Cell growth and multiplication: (a) the number of viable yeast cells per unit volume of liquid growth medium decreases as salt content increases, (b) the biomass of the culture (i.e., the total weight of yeast cells per unit volume of liquid growth medium) decreases as salt content increases, and (c) the length of the lag phase (i.e., the incubation period be-
between inoculation of the culture and detectable initiation of cell growth) lengthens as salt concentration increases. (ii) Utilization of the primary carbon and energy source is reduced. (iii) Change in concentration of metabolic products: (a) there is a decrease in the production of ethanol as salt content increases and (b) there is an increase in the concentration of other fermentation products (such as glycerol, acetaldehyde, etc.) as salt content increases.

MATERIALS AND METHODS

Fermentors. The fermentations were carried out in 1-liter Pyrex glass jars with approximately 600 ml of working volume. Closure was with aluminum foil. No stirring, aeration, or pH-control devices were used during the runs.

Growth medium. Maxon and Johnson Synthetic Medium C (6) was the basal fermentation medium. Sodium chloride was added as American Chemical Society-grade NaCl (catalog no. S-271, Fisher Scientific Co.). This liquid culture medium was converted to the semisolid mode by the addition of 16% (wt/vol) gelatin (BBL Microbiology Systems). The final fermentation medium contained 10% glucose.

Organism. The fermenting organism was S. cerevisiae (bakers’ yeast) purchased as Fleischmann’s dry yeast in foil packets. Each yeast package was used only on the day it was opened.

The inoculum for 600 ml of growth medium was prepared by the suspension of 1.2 g of dry yeast in 20 ml of sterile Maxon-Johnson Synthetic Medium C at room temperature.

Fermentation start-up. Maxon-Johnson medium (600 ml) with selected salt content (0.0 to 5.0% [wt/vol] NaCl) was poured into a fermentor covered with aluminum foil and steam sterilized at 15 pounds per square inch gauge for 30 min.

The unit was cooled to 60 to 65°C and gelatin was added. With the temperature held in this range, the contents of the fermentor were mixed with a magnetic stirrer until the gelatin dissolved. At this point the growth medium was adjusted to pH 5.0 with sterile 1.0 N HCl or 1.0 N NH₄OH solution.

The fermentor and contents were cooled to 40°C and the 20-ml suspension of yeast cells was added. The loaded fermentor was then placed in a 25°C constant temperature bath, and the mixture was stirred at 500 rpm to keep the cells dispersed while gelation took place. When gel formation was complete, stirring was stopped and fermentation was continued at 25°C.

pH monitoring. The pH of the fermenting mash was measured by using a miniature combination electrode (Sargent-Welch Scientific Co.) positioned about 5 cm below the surface of the growth medium and 2 cm from the fermentor wall. The probe was moved occasionally so that the tip would not be immersed in a trapped gas pocket, which would lead to faulty readings.

Sampling technique. After selected incubation periods, 20-ml samples of the semisolid fermenting mixture were removed by means of a sterile stainless steel spatula. Each sample was transferred to a 50-ml flask which was closed with a rubber septum to minimize the loss of volatile components. The sample was liquefied at 40 to 45°C. After being mixed thoroughly, a 1.0- or 2.0-ml portion of the melted sample was removed and diluted with warm distilled water in various proportions, depending upon the concentration of the component being measured and the sensitivity of the analytical method being employed.

Biomass of yeast cells. The concentration of yeast cells in the fermenting mash was measured by the turbidimetric (absorbancy) method. A 2.0-ml portion (1.0-ml portions in later stages of fermentation) of the liquefied sample described above was diluted 10-fold with 40 to 45°C distilled water in a test tube. Dilution was crucial to the separation of yeast cells from the gel matrix in the subsequent centrifugation at 1,800 rpm for 10 min. The supernatant cell-free culture medium was stored for determination of glucose, ethanol, acetaldehyde, glycerol, and L-lysine.

The cell pellet was washed with 10 ml of distilled water at 40 to 45°C, succeeded by a second centrifugation. The supernatant was discarded. Finally, the washed cells were suspended in 10 ml of distilled water. The optical density of the washed cell suspension at 610 nm was measured in a Bausch & Lomb Spectronic 20 spectrophotometer that had a red light filter.

A standard curve of optical density versus yeast dry weight (grams per liter) that covered the appropriate range of concentrations was made with a series of six suspensions prepared from the dry, packaged yeast cells.

Glucose determination. After appropriate dilution of the cell-free fermentation mash, glucose was measured by the Somogyi-Nelson method (4a) with optical density measurements at 425 nm in the Spectronic 20. The gelatin in the fermentation medium interfered with the assay. Correction for this interference was made by running the appropriate blank. Results were expressed as grams of glucose per liter of fermenting medium.

Ethanol determination. Ethanol in the diluted cell-free fermentation mash was estimated by the alcohol dehydrogenase method of Kaplan and Ciotti (5) with readings made in the Spectronic 20 at 340 nm. Results were expressed as grams of glucose per liter of fermentation growth medium.

Acetaldehyde determination. Assay of acetaldehyde was made on undiluted cell-free fermentation mash by using dehydrogenase (catalog no. 171-832) purchased from Boehringer Mannheim Biochemicals. Readings of NADH were made in the Spectronic 20 at 340 nm. Results were reported as grams of acetaldehyde per liter of fermentation mash.

Glycerol determination. Glycerol content of the diluted cell-free fermentation mash was measured by the glycerol kinase method (1) with the Boehringer Mannheim Biochemicals glycerol UV test kit. Readings were made in the Spectronic 20 at 340 nm. Results were recorded as grams of glycerol per liter of fermentation mash.

Lysine determination. The intracellular free L-lysine content of the yeast cells was measured by microbiological assay after extraction by boiling.

Lysine was extracted from an appropriate weight of yeast cells by suspending the cells in 5 ml of distilled water in a test tube and holding the suspension in a boiling water bath for 20 min. The cell debris was removed by centrifugation.

The microbiological assay used was that described in the Difco manual (4), with Pediococcus cerevisiae.
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The (NRRL B-1116) as the test organism. The assay was made on the extract at 37°C with incubation for 18 h. The final cell concentrations of the test organism were measured in optical density units in the Spectronic 20 at 660 nm. The lysine results were reported as specific free lysine, i.e., % (wt/wt) L-lysine per unit cell mass of yeast.

RESULTS AND DISCUSSION

Number of yeast cells per unit volume of liquid growth medium. Combs et al. (3) have studied the effects of changing NaCl levels on the multiplication of Candida albicans, as measured by conventional plate counts with Sabouraud agar. NaCl in 1.0% concentration has essentially no effect on the 96-h viable cell count. This is not surprising since 1.0% NaCl solution is essentially isotonic physiological saline solution. On the other hand, 3.5% NaCl reduces the 96-h count by 70 to 80%, and 5.0% NaCl by about 90%.

Biomass of yeast cells per unit volume of liquid growth medium. In the study of several yeasts isolated from marine environments, Ross and Morris (8) have found that as the concentration of NaCl in the culture medium increases, the production of yeast biomass decreases. Norkrans (7) has reported that 4.0% NaCl reduces the growth of S. cerevisiae by 10 to 15% compared with that of the control (0.0% NaCl), whereas 8.0% NaCl cuts the biomass to about 90% of the control level. These researchers measured biomass by the absorbancy (turbidimetric) method.

Combs et al. (3) have investigated changes in biomass by monitoring dry cell weight per liter after a 48-h incubation period. They have reported that biomass falls from 5.61 g/liter at 0.0% NaCl, to 3.40 g/liter at 1.0% NaCl, and to 1.90 g/liter at 5.0% NaCl. Umemoto et al. (18) have reported that the high concentrations of molasses electrolytes reduce yeast cell growth. Furthermore, they have showed that individual pure inorganic salts have a similar effect.

Tanner et al. (16) have found that 40 g of NaCl per liter reduces cell production in a semisolid (40 g of gelatin per liter) growth medium. Figure 1 shows results of our studies on the effects of NaCl on S. cerevisiae growth in gelatin. These kinetic trajectories show that inhibition of yeast cell growth occurs in semisolid fermentation as it does in liquid fermentation. At virtually every time point, the trend is clear; i.e., NaCl quantitatively reduces the cell content up to about 5% NaCl, where growth becomes negligible.

Length of lag phase. Ross and Morris (8) and Norkrans (7) have plotted absorbancy (as a measure of cell growth) versus incubation time. The early portions of their curves suggest that increasing NaCl content proportionately lengthens the lag period in the yeast growth curve in liquid culture. The curves in Fig. 1 show that similar inhibition occurs in semisolid cultures.

Utilization of primary carbon and energy source. During an investigation of the liquid alcoholic fermentation of Okinawa molasses, Umemoto et al. (18) have observed a so-called "sugar defect"; i.e., sugar in the molasses mash is not converted to alcohol in the expected yields based on glucose equivalents. The higher the concentration of electrolytes in the mash, the larger the "sugar defect," i.e., the poorer the "fermentation efficiency." A similar inhibitory action has been noted in liquid fermentations utilizing six different yeasts.

Spencer (9) has reported that glucose consumption by the yeast Saccharomyces rouxii is reduced by the presence of 3.1 M (18% wt/vol) NaCl in the liquid growth medium. Brown (2), studying S. cerevisiae, has found that 1.73 M (110% wt/vol) NaCl in liquid culture increases sugar utilization. Tanner et al. (14) have found that a concentration of NaCl as low as 0.3 M (ca. 1.5% wt/vol) initiates a reduction in the rate of glucose uptake for liquid cultures of S. cerevisiae.

It is interesting that when Umemoto et al. (18) added 10⁻³ M sodium azide to the growth medium, the growth of a yeast identified as alcoholic yeast Hakken no. 1 was strongly inhibited, whereas sugar uptake was only slightly reduced from that of the control, suggesting that the sugar is used less in cell synthesis than for the synthesis of noncellular products, such as glycerol.

In semisolid culture, an increase in the NaCl level in the growth environment above 2.0% markedly reduced the rate of glucose uptake by S. cerevisiae, although eventually (by 69 h) the
glucose was entirely used (Fig. 2).

Decrease in the concentration of ethanol produced. Tajima and Yoshizumi (10) have reported that as the concentration of NaCl in the growth medium increases from 0.0 M to 1.0 M, the amount of ethanol produced falls from 5.95 to 5.05 ml per 100 ml of liquid growth medium.

Results obtained in the present semisolid study (Fig. 3) support the results of Tajima and Yoshizumi (10) for salt levels above 2% up to 30 h. After 30 h, the differences tend to be negligible. Although initial ethanol production rates are slowed proportionately by increasing salt levels, it appears that ultimate ethanol content is essentially the same, regardless of salt concentration.

Increase in the concentration of other fermentation products. In 1967, Umemoto et al. (18) reported that not only do high concentrations of electrolytes in liquid culture cause the "sugar defect," but also high concentrations of electrolytes in several yeasts promote high accumulations of polyhydric organic compounds, such as glycerol, 2,3-butanediol, arabitol, and erythritol. Tajima and Yoshizumi (10) have found that Saccharomyces formosensis Nakazawa in 0.25 to 1.00 M NaCl converted much of the sugar substrate into glycerol, 2,3-butanediol, mannitol, erythritol, organic acids, vicinal diketone, acetaldehyde, and CO₂.

Our gelatin study results corroborate previous reports on the effect of salt in stimulating the production of glycerol during the fermentation of glucose by S. cerevisiae in liquid culture (Fig. 4). However, it should be noted that the control (0.0% NaCl) curves, after reaching a plateau, subsequently show a gradual drop in glycerol beginning at about 24 h, at which time the glucose substrate has been exhausted (Fig. 2), suggesting the shift to glycerol as substrate for the yeast cells. This suggestion is reinforced by the diauxie curves (Fig. 1) for low salt levels. The same sequence of events is seen in the 1.0, 2.0, and 3.0% NaCl curves, with the reduction in glycerol beginning after 30 to 35 h. The concentration of glycerol continued to build up in the 4.0 and 5.0% NaCl cultures in which (Fig. 2) glucose was not exhausted during the length of the run.

Brown (2) has discussed evidence for considering the polyols synthesized by S. cerevisiae as fulfilling several physiological functions in the yeast, including the role of food reserves.

The acetaldehyde curves (Fig. 5) corroborate the previously observed (10, 20) increasing buildup of acetaldehyde in liquid cultures at high NaCl levels, but only if measurements are made between 10 and 17 h or after 40 h. All of the curves, including the control curve, reach a maximum and then fall off at variable rates.

Tempest et al. (17) have reported that the presence of 4% (ca. 0.67 M) NaCl S. cerevisiae-
The rise in pH of the cultures containing high levels of NaCl is shown in Fig. 7. The increased acidity at lower salt concentrations corresponds to higher cell levels (Fig. 1). What is particularly interesting, however, is the fact that a micro pH probe can be used for on-line pH monitoring of cells growing in a gel system, as well as in a liquid culture.

Mechanisms proposed to explain the inhibitory effects of NaCl on yeast fermentations. Umemoto et al. (19) have suggested a partial explanation of the inhibitory effects of electrolytes in terms of the inhibition of yeast (pyruvate) carboxylase, the enzyme that catalyzes the decarboxylation of pyruvate to acetaldehyde. Obviously, if acetaldehyde production is slowed down, the production of ethanol is reduced proportionately.

They concluded that in the absence of acetaldehyde, if the fermentation was to continue, a hydrogen acceptor other than acetaldehyde must become available to oxidize the NADH...
formed during the metabolism of glucose in the Embden-Meyerhof pathway. It was proposed that the substitute hydrogen acceptor was phosphoglyceric acid, hence the subsequent production of excess glycerol.

The work of Tajima and Yoshizumi (11) shows no early (24- to 48-h) inhibition of acetaldehyde production by 1.0 M (6%) NaCl. Figure 5 also shows rapid production of acetaldehyde in the early stages of glucose fermentation in the presence of 3.0, 4.0, and 5.0% NaCl. There is inhibition with eventual recovery at the 1.0 and 2.0% NaCl levels.

Tajima and Yoshizumi (11) have suggested that ethanol production is reduced and acetaldehyde accumulation is increased under highly salted growth conditions because the salt inhibits alcohol dehydrogenase (the enzyme which reduces acetaldehyde to ethanol). This would explain the lengthening of the lag phase in the salt cultures in Fig. 3. This is consistent with the rapid, early accumulation of acetaldehyde in the curve for the 5% NaCl cultures shown in Fig. 5. It would also account for the fact that the ethanol production rate (which is proportional to the alcohol dehydrogenase activity) is significantly less in the kinetics for late times compared with early times in the hysteresis curve (Fig. 8). The direction of the kinetic hysteresis curve has been shown to be useful as an indicator of a decay in enzyme activity (13).

The relationship between the early and late time enzyme activities becomes even more pronounced when the area of hysteresis curve (another measure of the difference between the upper and lower trajectories) is graphed as a function of the salt level in Fig. 9. This new parameter indicates greater alcohol dehydrogenase decay with time as the NaCl level increases.

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LITERATURE CITED


