Osmoregulation Mechanisms of the Yeast *Sporidiobolus salmonicolor*

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The osmoregulation process of the yeast *Sporidiobolus salmonicolor* was studied when it was subjected to a shift in osmotic pressure. The nature of the solute used to obtain an a_w value close to 0.97 was studied, particularly its ability to cross the cell membrane and to be metabolized by the yeast. When glucose was present in the medium, solutes acted only as osmotic agents. At low water activity, glycerol was synthesized and accumulated by the cell to restore the thermodynamic equilibrium; an increase in osmotic pressure also induced an increase in the total specific production of γ-decalactone. When glycerol and mannitol were used as the carbon sources in the medium (without the addition of glucose), the specific aroma production was increased as compared to that obtained in the media with glucose.

INTRODUCTION

When placed in a hypertonic medium, the thermodynamic equilibrium of a cell will be restored by a passive exit of water. This flow of water concerns only free water which is osmotically active (between 60 and 80% of the total weight of water) and not that which is bound to biological molecules and therefore osmotically inactive (Levin, 1979). This exit of water results in plasmolysis of the cell, which could injure or kill the cell by the mechanical denaturation of membrane enzymatic systems, particularly those involved in energetic pathways (Gervais, 1988).

The first stage of the passive water mass transfer occurs in a few seconds after the osmotic shift (10–20 s for the total exit of water). A second stage, previously described (Zimmermann, 1978; Steudle et al., 1983), corresponds to an active response of the cell to the loss of water. This stage is certainly initiated through the activation of enzymatic systems by a low level of osmotic pressure as previously demonstrated (Grajek and Gervais, 1987). These enzymatic systems often lead to the synthesis of low molecular weight molecules which could either increase the inner osmotic pressure or modify the membrane permeability. These compatible solutes can be of different chemical natures such as polyols or sugars, often described for yeasts, algae, and fungi, and ions and amino acids for bacteria. This second stage, which involves an important synthesis of solutes, is longer than the previous phase, and the survival of the cells could depend on the time constant of these syntheses.

Nonosmotolerant cells such as *Saccharomyces cerevisiae* synthesize great quantities of polyols which continuously cross the membrane following the chemical potential gradient. On the contrary, osmotolerant microorganisms synthesize lower quantities of polyols but simultaneously modify the membrane permeability to these polyols to maintain a very high concentration gradient, between intra- and extracellular media, up to 8000 (Brown, 1978). In previous works, the effect of osmotic pressure level on the γ-decalactone production of *Sporidiobolus salmonicolor* has been studied (Gervais and Battut, 1989; Gervais and Pécot, 1991), and it has been assumed that the production of this aroma compound, although related to osmoregulation, was only a secondary phenomenon (Gervais, 1990).

The aim of the present study was to explain the osmoregulation process of *S. salmonicolor* when subjected to a shift in osmotic pressure. The nature of the solute used to decrease the a_w level of the medium has been studied, particularly its ability to cross the cell membrane and be metabolized by the yeast.

MATERIALS AND METHODS

Media and Water Activity Regulation. A synthetic culture medium was used as previously described (Gervais and Battut, 1989). The carbon source was generally glucose (30 g L^{-1}) except in one experiment in which glucose was substituted by glycerol or mannitol. To obtain an a_w value of about 0.97, four solutes were used: glycerol, mannitol, poly(ethylene glycol) (PEG) 600, and sodium chloride. The required concentration of each solute was calculated by applying Norrish's equation (Norrish, 1966) and using the corresponding mass value as shown in Table I. To maintain the same concentration of nutritive substances, the solution of depressor was incorporated in the medium as a replacement for the usual volume of water. The a_w values were measured using an osmometer (Roebling, Germany).

Microorganism. The yeast used was *S. salmonicolor* (Fell and Talman, CBS 2686), which produces a characteristic fruity odor. The aroma compounds have been identified by Tahara et al. (1972, 1973). Among the present lactones, γ-decalactone has a specific peachy odor.

The strain was kept on malt extract agar at 4 °C.

Cultivation Conditions. Cultures were made in 250-mL Erlenmeyer flasks containing 100 mL of culture medium. Steam sterilization was done at 121 °C for 20 min. The inoculation was performed using a suspension of yeasts cultivated (between 2 and 3 days) on malt extract agar (MEA) gelose slants. Yeasts were cultivated for 3 days in a medium without solute (a_w close to 1). Aeration was realized by agitating the flasks at 250 rpm. The inoculum was adjusted (after counting) to obtain an initial concentration of about 10^5 cells/mL in each Erlenmeyer flask. Incubation was done at 24 °C and 250 rpm with a rotary shaker (Infors, Switzerland). Fermentation proceeded for 15–20 days with regular analysis.

Measurements. *Water Activity Measurements.* The values of the fixed a_w in the different cultivations were verified at the beginning and the end of the cultures with a hygrometer (Sinascope, Switzerland) and an osmometer (Roebling). Only very slight a_w variations, less than 0.01, were found due to the production of metabolites and water.

Biomass. Biomass was evaluated by measuring the optical density at 610 nm. A specific calibration was made for this experiment to link OD and biomass. The linear regression between the OD and cell counts was found to be highly significant ($r^2 = 0.895$). A dilution was sometimes necessary to give values in the zone of linearity (OD between 0.1 and 0.7).

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Table I. Mass of Depressors Added to the Medium To Obtain an a_w Value of About 0.97 Calculated with the Norrish Equation

depressor	mol mass, g	mass of depressor added, g L^{-1}: media without glucose	mass of depressor added, g L^{-1}: media with glucose[a]
glycerol	92.06	153.01	133.31
mannitol	182.17	304.64	273.40
PEG 600	600.00		492.83
NaCl	58.44		68.37

[a] Concentration of glucose added: 30 g L^{-1}.

Aroma Analysis. (*a*) *Aroma Extraction and Analysis.* The γ-decalactone concentration was determined after an extraction–concentration process was applied using the following method: 50 mL of the culture medium was centrifuged for 15 min at 4000 rpm, and 0.5 mL of a solution of γ-undecalactone (internal standard) at 400 ppm (v/v) was added to 40 mL of the supernatant. This solution was then filtered through a separative liquid chromatographic nonpolar column (Sep-Pak C18, Waters Associates, Inc., Milford, MA). The column was then eluted with 1.5 mL of hexane; 2 μL of the extracted solution was injected into the gas chromatograph Packard 437A with a flame ionization detector under the conditions previously described (Gervais and Battut, 1989). The flow rate values were as follows: hydrogen flow rate, 25 mL/min; nitrogen flow rate, 16.5 mL/min; air flow rate, 220 mL/min.

The chromatograph was combined with a Shimadzu CR3A integrator for the analysis of the peak areas. Each concentration was the average result of three injections.

A calibration was previously made with the internal standard to obtain the initial concentration of γ-decalactone in the sample.

(*b*) *Determination of Intracellular Aroma Content.* To identify and quantify the cellular concentration of aroma, cells were ground in a rotary cell homogenizer MSK (B-Braun, Germany): 15 mL of cellular suspension was added to 1.25 mL of a solution of γ-undecalactone as the internal standard (400 ppm v/v), and this was then placed with 40 g of small glass beads (of diameter 0.17–0.18 mm) in a Duran glass bottle. This bottle was placed inside a steel tube, with an axis which moved in circular motion at a speed of 4000 rpm for 4 min. Shocks between the cells and beads led to disruptions of the cells. To prevent an increase in temperature, the material was cooled by liquid CO_2, and the temperature was thus maintained below 20 °C. After the grinding process, the glass beads were removed from the homogenate and washed with distilled water. The aroma content was determined by chromatographic analysis as previously described. Results were corrected from a percentage of waste (44%) due to the grinding process as previously reported (Gervais and Pécot, 1991).

Glycerol and Glucose Analysis. Enzymatic tests (Boehringer, Mannheim, Germany) were used to measure glycerol and glucose inside and outside the cells. The determination was realized both in the homogenate obtained after the cells had been ground and in the supernatant (after centrifugation of the growth medium), after the samples were placed in a water bath at 80 °C for 15 min to stop the enzymatic reactions.

RESULTS

Estimation of the Osmotic Effect of a Solute Addition on Yeasts. *Glucose Repression on Glycerol Consumption.* A shift in the osmotic pressure of the cultivation medium is generally obtained through the addition of low molecular weight solutes, such as polyols or salts. However, some polyols such as glycerol or mannitol could be used as carbon sources by the cells as well as glucose.

In an initial experiment, glycerol was added to the cultivation medium at a concentration of 4 g L^{-1}. Results (proposed in Figure 1) show that glycerol consumption began only on the 12th day of the cultivation, when the glucose concentration was very low. This experiment concludes that glycerol consumption was repressed by high glucose concentrations in the medium as previously described for yeasts (Brown, 1975). So, in the presence of glucose, glycerol acts only as an osmotic agent during the first stage of the cultivation.

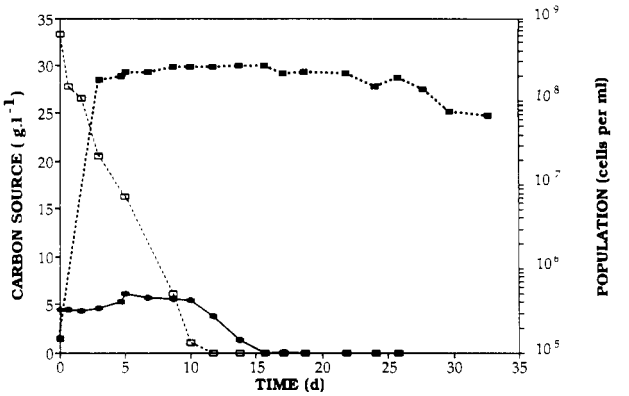

Figure 1. Kinetics of population and glucose and glycerol concentrations during a cultivation in a medium containing 4 g L^{-1} glycerol. Population was correlated with OD measured at 610 nm; glycerol and glucose were determined enzymatically. (- -■- -) Population; (- -□- -) glycose; (—■—) glycerol.

Table II. Evaluation of Mean Maximal Population and Aroma Production in Different Media at a_w = 0.97 after 12 Days of Cultivation[a]

depressor	maximal population, 10^8 cells/mL	γ-decalactone production, mg/10^{10} cells
glycerol	2.1 (±0.7)	0.26 (±0.06)
mannitol	1.5 (±0.2)	0.30 (±0.03)
PEG 600	1.1 (±0.3)	0.28 (±0.05)
NaCl	0.8 (±0.8)	0.25 (±0.09)
control, $a_w \simeq 1$	2.0 (±0.2)	0.10 (±0.05)

[a] Confidence interval at 0.95 level is given in parentheses.

γ-Decalactone Production by S. salmonicolor. In previous experiments (Gervais and Battut, 1989) concerning this aroma production, glycerol was the only solute used to increase the osmotic pressure (or to decrease the water activity of the medium). In this case, three other solutes were used, and their effects on aroma production in glucose media were compared to the effect of glycerol, for about the same level of a_w (0.97).

Biomass evaluation and aroma production in these different media, after 12 days of cultivation, are proposed in Table II. For each treatment, the results correspond to the mean value of three independent cultivations. The effects of the solutes on biomass production were different; PEG 600 and NaCl led to a smaller biomass than glycerol and mannitol. This could be explained by a toxic effect of NaCl and PEG 600 previously described (Chirife and Ferro Fontan, 1980). For glycerol and mannitol, biomass production was very close to that produced in a normal medium at $a_w \simeq 1$ (about 2.10^8 cells/mL) as shown previously (Gervais et al., 1988).

For γ-decalactone production, the total specific productivities were very close regardless of the solute used. These values were significantly higher than the productivity observed in a normal medium (2.5–3 fold higher). The nature of the solute in this case had no influence; only the osmotic effect was acting.

From these results and to optimize the mass of lactone biosynthesized during a cultivation, both effects (on biomass and aroma) must be considered. In this case, mannitol and glycerol addition will significantly increase such a biosynthesis in comparison with PEG or NaCl addition or control media (about 3-fold more).

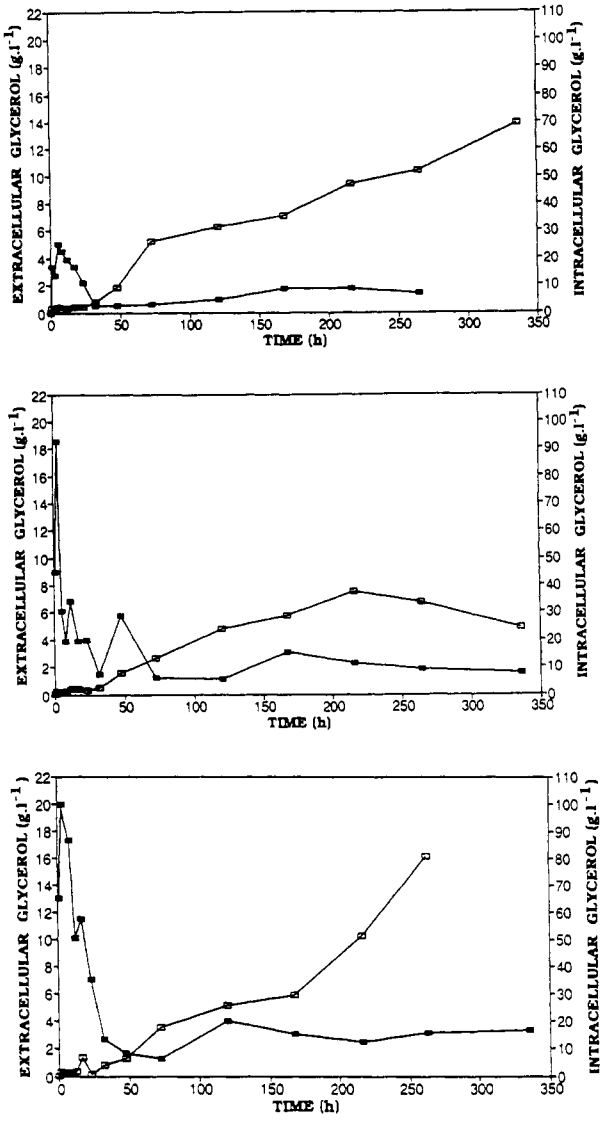

Figure 2. Kinetics of production and intracellular accumulation of glycerol at $a_w = 0.97$ adjusted with mannitol (a, top), PEG 600 (b, middle), and NaCl (c, bottom). (■) Intracellular glycerol; (□) extracellular glycerol. Intracellular glycerol was determined after separation of the cells from the medium and after the cells had been ground.

Glycerol Production by S. salmonicolor. During these experiments, the kinetics of glycerol production by the yeasts has been studied in the intra- and extracellular media at an a_w close to 0.97, using PEG 600, mannitol, and NaCl.

The first qualitative result is that glycerol is effectively an osmotic solute which is synthesized by *S. salmonicolor* to restore its internal thermodynamic equilibrium.

Quantitative results are proposed in Figure 2. To understand the osmoregulation mechanisms of the yeasts, it is interesting to compare the intra- and extracellular content of glycerol. It was found that glycerol was rapidly accumulated in large quantities in the cell during the first 10 h before diffusing into the external medium. Such a result could be related to the assumption of a two-phase osmoregulation process. Nevertheless, the glycerol diffusion into the external medium was not sufficient to explain the increase in the external glycerol concentration. So, continuous synthesis and excretion of this solute during the culture could be responsible for this increasing concentration.

Large variations in the glycerol gradient between the intra- and extracellular media (represented in Figure 3)

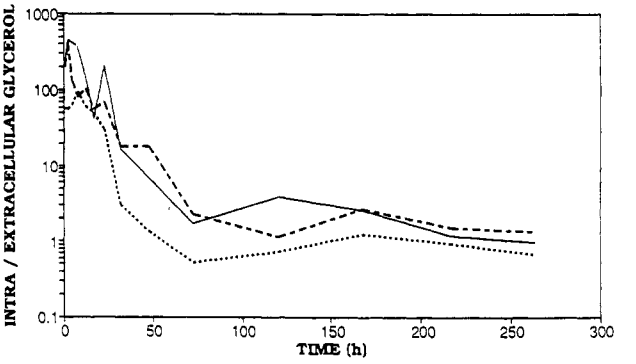

Figure 3. Kinetics of the glycerol gradient between intra- and extracellular media in cultures of $a_w = 0.97$, with three different depressors. (- - -) Mannitol; (– – –) PEG 600; (—) NaCl.

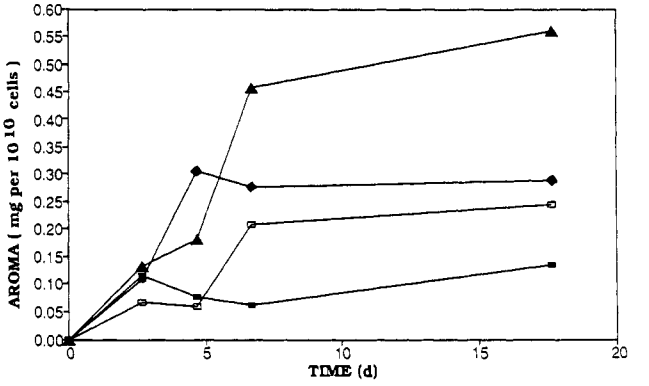

Figure 4. Influence of the nature of the added solute on the specific production of γ-decalactone by the cell at $a_w = 0.97$ (intracellular aroma + extracellular aroma). Aroma was measured by chromatographic analysis after separation from the medium on a nonpolar column. (■) Glucose + glycerol; (□) glycerol; (◆) glucose + mannitol; (▲) mannitol.

can be observed. In the first stage (20 h), the gradient was very high (50–500), corresponding to an accumulation of glycerol in the intracellular medium. After 25 h, the gradient decreased until it reached the equilibrium value (0.7–1.6) for the last stage of cultivation. It can be noted that the final ratio was below 1 for mannitol, which could diffuse across the membrane and so act as an osmoregulator, equal to 1 for NaCl, and greater than 1 (1.61) for PEG 600, which could not enter the cell.

Estimation of the Metabolic Effect of a Polyol Addition on Yeasts. Mannitol and glycerol can be metabolized by yeasts in the absence of glucose. So, to discriminate between the osmotic effect and a possible metabolic effect, these solutes were used as carbon sources for the cultivation of *S. salmonicolor*. These experiments were realized at an a_w close to 0.97, with and without the addition of glucose.

Results showed that specific activity in γ-decalactone production for the different solutes, which is proposed in Figure 4, was greatly increased for the media without glucose. Specific aroma production was 2-fold higher for mannitol than for mannitol–glucose media at $a_w = 0.97$; for this same a_w value in the glycerol medium, the specific aroma production was 0.75-fold higher than in the glucose–glycerol medium.

So, the use of mannitol or glycerol as a carbon source by *S. salmonicolor* led to metabolic changes. Some morphological modifications have also been observed in these experiments. In the media without glucose, hyphal formation and cell aggregation were noticed.

DISCUSSION

Osmoregulation Process. Shifts in the osmotic pressure of a cultivation medium, caused by the addition of solutes, can induce thermodynamic rearrangement of the cells, as well as metabolic modifications, if the solutes can be assimilated by the yeast.

For *S. salmonicolor* cultivated in glucose–mannitol medium, the osmotical equilibrium was provided mainly by glycerol synthesis.

Related to the volume of a normal cell (about 130 μm^3), the maximum internal concentration of glycerol contributed to 70% of the modification of the inner osmotic pressure necessary to equilibrate the external shock. So it could be envisaged that the synthesis of another solute such as arabitol (Adler et al., 1985) or more probably a persistant decrease in the cell volume occurred after the osmotic shift. It has been shown for other species that such a shift could lead to a decrease of about 30–40% of the cell volume, which could persist over the time of experiment. This modification is due to a change in turgor pressure, which became weaker as the osmotic pressure was increasing (Rose, 1976; Griffin, 1981).

Such a decrease in cell volume after a step of osmotic pressure has been previously observed (Niedermeyer et al., 1977; Morris et al., 1983). In this case, the observed glycerol production could explain the whole osmotic equilibrium of the yeasts.

The osmoregulation process involved the synthesis of glycerol with great variations in the concentration gradient between intra- and extracellular media over time. These variations allow the assumption to be made that the cell permeability was modified during the osmoregulation process and that physical or chemical modifications of the plasma membrane were involved in such a process.

The time constant of the water flow over the cell, which lies between 10 and 20 s, must be compared to the greater time constant of the glycerol synthesis (6–7 h, see Figure 2). So, for the duration of a few hours the cell will stay in a nonequilibrated medium. In such a case, two parameters will be involved in the survival of the cells: the intensity of turgor pressure, which will act as a thermodynamic tank, and the modification of the membrane to prevent water exit.

When the yeasts are cultivated in mannitol or glycerol media, it could be assumed that the osmotic equilibrium is realized mainly by the diffusion of polyols across the plasma membrane. The time constant of this diffusion phenomenon would be obviously shorter than the time constant of the previous synthesis phenomenon. So, in this case the cell will equilibrate its inner osmotic pressure faster than in the glucose medium and the energetic consumption intending to reach this equilibrium will be lower.

Aroma Production. The increase in specific activity of γ-decalactone by *S. salmonicolor*, related to the increase in the osmotic pressure of the external medium, could be assumed to be a secondary consequence of the osmoregulation process. Indeed, the time constant of this synthesis was very important (5–7 days) with regard to the previous phenomena, and the synthesized quantities were too low to significantly modify the osmotic pressure of the cell.

Nevertheless, the γ-decalactone synthesis could be related to the glycerol or mannitol concentration in the cell. Indeed, previous works have shown that, in media of high osmotic pressure, both glycerol and mannitol degradation cycles were involved for algae (Ben Amotz and Avron, 1981) and filamentous fungi (Hult and Gatenbeck, 1978).

These cycles seem to be initiated by a high osmotic pressure corresponding to a high internal concentration of glycerol or mannitol. Both cycles induced a large reduction in the quantity of the coenzyme NADPH (reduced nicotinamide adenine dinucleotide phosphate), which is involved in lipid biosynthesis.

In the same way an increase in the osmotic pressure was found to induce a diversion of acetyl coenzyme A from the Kreb's cycle toward the biosynthesis of lipids (Mattiasson and Hahn-Hägerdal, 1982). This phenomenon allows the increase of compounds derived from lipid metabolism to be explained in osmotolerant microorganisms. Thus, Ben Amotz and Avron (1981) have noticed the increase in β-carotene production by *Dunaliella* cultivated at high osmotic pressure levels.

γ-Decalactone was also derived from lipid anabolism (Boldingh and Taylor, 1962). The decanoic acid, which is the direct precursor of γ-decalactone in *S. salmonicolor*, can be obtained from successive methylations of acetyl coenzyme A. Then, from Tressl et al. (1978), γ-decalactone was obtained after γ-hydroxylation of the decanoic acid.

So, when cultivated in hypertonic media, *S. salmonicolor*, which synthesizes or accumulates glycerol, could initiate such a cycle. In the presence of glucose, the added glycerol is not metabolized and the coenzyme production could only be initiated by the synthesized glycerol. This would explain the observed constant specific productivity in γ-decalactone whatever the solute used as shown in Table II.

In the presence of glycerol or mannitol as the only carbon sources, these solutes can penetrate the cell and the NADPH production will be realized from the exogen polyols. This phenomenon would explain the observed increase of the γ-decalactone biosynthesis in such cases.

ACKNOWLEDGMENT

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Registry No. Glycerol, 56-81-5; glucose, 50-99-7; γ-decalactone, 706-14-9; mannitol, 87-78-5.