Growth of Osmotolerant Yeasts at Different Water Activity Values

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ABSTRACT

The influence of water activity (a_w) on growth (lag-phase, mean generation time and cell yield) of osmotolerant yeasts was determined by culturing 7 strains in broths at 10 different a_w values in the range of 0.998 to 0.626 and by counting the Colony Forming Unit (CFU) per ml. Broths were adjusted to the desired a_w by means of fructose. None of the tested strains could grow at a_w 0.701 and 30°C. During 60 d of incubation at this a_w and temperature, slight reductions of the initial CFU/ml counts were noted. By incubation at a_w <0.701 these reductions were larger. Six strains of Zygosaccharomyces rouxii grew at a_w 0.760, whilst a strain of Zygosaccharomyces bailii could not grow at a_w <0.858. For six strains the optimum a_w for growth was in the range of 0.958 to 0.998. A single strain of Z. rouxii showed optimum a_w for growth in the range of 0.913-0.958. Therefore, it was appropriate to redefine it as "osmophilic". Because of high dehydration, cells actively grown at a_w 0.837 were approximately 30% smaller than cells actively grown at a_w 0.998.

A detailed discussion of water activity (a_w) is outside the scope of this introduction, but further information can be found (7,8,34,41). The a_w of the substrate defines the amount of available water, and it therefore has a large influence on microbial growth. Maximum growth does not take place at a_w 1.00 but at a lower optimum point, dependent on the specific strain. In general, lowering the water activity value of the growing medium under optimum conditions results in a decrease in the growth rate. When it reaches "zero", the microorganisms either die or survive in a dormant condition.

Several authors have proposed definitions of osmotolerance; most are based on the ability to grow at a particular sugar concentration or a_w. There is some disagreement, however, about the characteristics of osmotolerant yeasts (2,5,6,18,22,32,43). In the treatise of Kreger-van Rij (13), which represents the newest position on yeast taxonomy, the osmotolerance is tested by the ability to grow on agar containing 50% (w/w) glucose. This will also be adopted in the present work; nevertheless, yeasts capable of growing in 50% (w/w) glucose (a_w 0.909 at 30°C) have been defined as "osmotolerant" and those growing in 60% (w/w) fructose (a_w 0.837 at 30°C) have been called "highly osmotolerant".

Several experimenters have carried out trials to obtain tolerance values for growth of osmotolerant yeasts. Since these values have been expressed using many parameters, but not a_w-value tolerance, great caution is needed in reconciling old statements with recent ones. For many investigators, water activity was temperature-independent, so no particular attention was given to its measurement. The lowest tolerance value for growth as recorded at present, is 62% relative humidity - a_w 0.62 - (33). From 1950 to 1986, with only two exceptions (14,39), such a low tolerance has not been recorded. Tilbury (39) detected growth of Zygosaccharomyces rouxii at a_w 0.65 and Leveau and Bouix (14) found strains of Zygosaccharomyces bailii and Z. rouxii able to grow at a_w 0.65 and a strain of Zygosaccharomyces bisporus, which grew at a_w 0.66. Conversely, several authors demonstrated that a_w values such as those mentioned above were too low and inhibited growth of the yeast strains examined (1,9,15,16,21,25,28,31).

Most previous trials of a_w-tolerance for growth have been done with type strains only, which were probably maintained for a long period on agar slants or in the lyophilized state, although loss, gain or lack of variance of osmotolerance during culture storage have not yet been fully investigated. Moreover, differences in the biological effects of humectants at equal a_w have been reported (35). Thus Tilbury (40) concluded that spoilage potential of a given food at a particular a_w depends on the nature of the humectant and other food ingredients.

It was the purpose of the present trials to investigate the a_w-tolerance for growth of a large number of yeast strains immediately after their isolation from high-sugar products, to avoid any influence that a long storage could have on osmotolerance. The influence of the water activity on the cell volume of actively growing yeasts was also investigated.

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TABLE 1. Composition, \(a_w\) and pH of different yeast extract fructose (YEF) broths.

<table>
<thead>
<tr>
<th>Property</th>
<th>YEF2</th>
<th>YEF5</th>
<th>YEF10</th>
<th>YEF30</th>
<th>YEF45</th>
<th>YEF60</th>
<th>YEF65</th>
<th>YEF70</th>
<th>YEF75</th>
<th>YEF80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose % (w/w)</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>30</td>
<td>45</td>
<td>60</td>
<td>65</td>
<td>70</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>(a_w) (30°C)</td>
<td>0.998</td>
<td>0.995</td>
<td>0.983</td>
<td>0.958</td>
<td>0.913</td>
<td>0.837</td>
<td>0.795</td>
<td>0.760</td>
<td>0.701</td>
<td>0.626</td>
</tr>
</tbody>
</table>

\*All media received 0.5% (w/w) yeast extract and were adjusted to pH 4.5 (see text).

MATERIAL AND METHODS

Preparation of media and diluents

All the media: yeast extract glucose 30 (YEG30) broth \([a_w 0.963; \text{pH} 4.5; 30\% (w/w) D-glucose, pure, Bender and Hobin, Zürich; 0.5\% (w/w) yeast extract, BBL. No. 11929); yeast extract glucose 30 (YEG30) agar \([a_w 0.960; \text{pH} 4.6; 30\% (w/w) D-glucose; 0.5\% (w/w) yeast extract; 1.5\% (w/w) agar, Technical No. 3, Oxoid No. 1.13], yeast extract glucose 50 (YEG50) agar \([a_w 0.906; \text{pH} 4.05; 50\% (w/w) D-glucose; 0.5\% (w/w) yeast extract; 1.5\% (w/w) agar] as well as the diluent DS30 \([a_w 0.958; \text{pH} 4.7; 30\% (w/w) D-glucose; 0.85\% (w/v) NaCl, reagent grade, Merck No. 6404; 0.1\% (w/v) peptone, trypsin digested, Merck No. 7213] were made by dissolving the chemicals in distilled water in a steam-boiler at 90°C and by autoclaving at 110°C for 15 min. The same procedure was used for preparing the yeast extract fructose (YEF) broths listed in Table 1.

Physical methods

\(a_w\) Measurement. All measurements were made after sterilization, using a hygrometer Model \(a_w\)-Box with measuring station EEJA manufactured by Novasina AG, Zürich. The values were obtained at 30°C after calibration of the equipment at 20°C.

pH-Adjustment. After sterilization, the pH of all YEG and YEF broths was adjusted to pH 4.5 with filter-sterilized 85% orthophosphoric acid. No pH-adjustment of agars and diluents was done. All pH-values were measured at 30°C with a pH-meter model Knick.

Microbiological methods

Pre-enrichment, preparation of the inocula and inoculation. YEG30 broth was inoculated from a maintenance culture on an agar slant (YEG50 agar) and incubated at 30°C for 60 h. The yeast was then harvested by centrifugation (Heraeus Centrifuge, Osterode, GFR; Relative Centrifugal Field (RCF) 2060 \(\times g\); 5 min) and resuspended in diluent DS30. Inoculation suspensions \((10^5 \text{ cells/ml})\) were prepared by counting with a Helber counting chamber and appropriate dilution with diluent DS30. Inoculation was done by transferring the inoculation suspension to culture broths, to attain an initial yeast count of \(10^3 \text{ cells/ml}\).

Incubation of the cultures. All liquid cultures were incubated on a rotary shaker (Clime-O-Shake, System Kühner AG, Basel) at 80 rpm and 30 ±0.1°C.

Colony Forming Units (CFU) counts. Counts of CFU/ml were carried out by plating serially diluted (DS30) portions on YEG30 agar. A single dilution series was prepared, followed by duplicate surface plating. The cultures were incubated for 3 to 5 d in an air-circulating cabinet at 30±0.5°C, with petri-dishes in plastic bags. The weighted arithmetical means of yeast colony counts were calculated and transformed into log number CFU/ml.

Relationship between \(a_w\), optical density and cell volume

Yeast taxa. Z. rouxii strain LMZ 100 and LMZ 105 as well as Z. bailii LMZ 108, previously isolated from high-sugar food products and maintained for 1 month on YEG50 agar slants at 4°C, were used in this investigation.

Cultures. Two-ml inoculation suspension were transferred to 198 ml each of YEF2, YEF10, YEF30, YEF45 and YEF60 broths. The cultures were incubated and at regular intervals growth development was determined \((a)\) by measuring the change in optical density OD at 610 nm (spectrophotometer Uvikon 810-820, Kontron Analytical, Zürich, \(b\) by total yeast microscopic counts and \(c\) by CFU/ml counts. In addition, the cell volumes at different \(a_w\) were calculated using the formula proposed by Mitchinson (19):

\[V = (\pi \times a \times b^2)/6\]

where \(a\) and \(b\) represent the average values for length and width of 100 cells observed under the microscope.

Cultural determination of the growth at different water activity values

To find minimum and optimum \(a_w\)-values, growth of 7 freshly isolated osmotolerant yeast strains was investigated. The behavior at different \(a_w\) values was determined by CFU/ml counts.

Yeast taxa. Z. rouxii strains LMZ 100, LMZ 105, LMZ 111, LMZ 114, LMZ 120 and LMZ 127 as well as Z. bailii strain LMZ 108, previously isolated from high-sugar food products and maintained for 1 month on YEG50 agar slants at 4°C, were used in this investigation.

Cultures. With each strain, 198 ml each of YEF2, YEF10, YEF30, YEF45, YEF50, YEF60, YEF65, YEF70, YEF75 and YEF80 with \(a_w\) values in the range of 0.998 to 0.626 were inoculated and incubated for 60 d. With strains LMZ 111, LMZ 114, LMZ 120 and LMZ 127 YEF5 broth \((a_w 0.995)\) was additionally investigated. At regular intervals counts of CFU/ml were taken. Preparation of the inoculum, inoculation and incubation as well as CFU counts were carried out as previously described. Thus a total of 67 cultures was investigated.

Determination of lag-times, mean generation times, cell yields and D-values. Calculation of lag- and mean generation \((g-)\) times was performed on a Hewlett-Packard 9805A calculator preprogrammed for linear regression analysis. G-values were calculated by using those data points which were judged to be in the exponential phase of growth, i.e. when there was a linear relationship between the log CFU/ml and the incubation time. The points were used in the regression analysis to calculate the line of best fit. The mean generation time was then calculated through the slope of this line. The lowest yeast count level was then substituted in the equation of the line of best fit to calculate the lag-time. \(N_{max}\) (maximal yeast yield) was extrapolated from growth curves. Decimal reduction values (D-
values for \( a_w \)-inactivation (representing the time necessary to reduce the CFU/ml counts by 90% and quantifying any loss of viability due to \( a_w \) stress) were determined by linear regression analysis, using all data points which were considered to be in a decreasing phase of the CFU/ml counts.

**RESULTS**

**Relationship between \( a_w \), optical density and cell volume**

In general, microscopically determined cell numbers (n) were slightly higher than CFU/ml counts. Formation of short chains or bushy clusters, appearing most often at the end of the log-phase and beginning of the stationary phase of growth, was observed. Since the relationship between n and OD was identical for all the tested strains, only the data connected with strain LMZ 100 are shown. Figure 1 illustrates the reduction in cell volume when the yeasts were grown at reduced \( a_w \) and shows the plot of optical densities of cultures grown at different \( a_w \)-values until a population of \( 2 \times 10^6 \) cells/ml was reached. Cells actively grown at \( a_w \) 0.837/fructose were approximately 30% smaller than cells actively grown at \( a_w \) 0.998/fructose.

**Minimum and optimum \( a_w \) for growth**

Investigating the behavior of 3 yeast strains at 9 different \( a_w \) values (0.998, 0.991, 0.958, 0.913, 0.837, 0.795, 0.760, 0.701 and 0.626) and that of 4 strains at 10 different \( a_w \) values (the above mentioned and 0.995), 67 curves were obtained. For each of the 67 curves, all growth parameters (lag-time, mean generation time and \( \log N_{\text{max}} \)) as well as the decimal reduction values D for \( a_w \)-inactivation were calculated. They are listed in Table 2a to Table 2g. The 67 curves have been classified into three types: normal growth curve (type 1), growth curve preceded by a decrease of population (type 2) and inactivation curve (type 3).

![Graph](image)

**TABLE 2a. Growth parameters (lag-time, mean generation time and \( \log N_{\text{max}} \)) as well as inactivation value D of Z. bailii strain LMZ 108 dependent on the \( a_w \) of the substrate.*

<table>
<thead>
<tr>
<th>Fructose (% w/w)</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>65</th>
<th>70</th>
<th>75</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_w ) (30°C)</td>
<td>0.998</td>
<td>0.995</td>
<td>0.983</td>
<td>0.958</td>
<td>0.913</td>
<td>0.837</td>
<td>0.795</td>
<td>0.760</td>
<td>0.701</td>
<td>0.626</td>
</tr>
<tr>
<td>Lag-time (h)</td>
<td>18.6</td>
<td>nt</td>
<td>14.4</td>
<td>6.1</td>
<td>12.7</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-Time (h)</td>
<td>1.1</td>
<td>nt</td>
<td>1.5</td>
<td>3.7</td>
<td>9.3</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>( \log N_{\text{max}} )</td>
<td>7.51</td>
<td>nt</td>
<td>7.67</td>
<td>7.11</td>
<td>6.82</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>nt</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>574</td>
<td>176</td>
<td>85.5</td>
<td>63.8</td>
<td>57.7</td>
</tr>
</tbody>
</table>

*nt = not tested; ng = not grown; ni = not inactivated.

**TABLE 2b. Growth parameters (lag-time, mean generation time and \( \log N_{\text{max}} \)) as well as inactivation value D of Z. rouxii strain LMZ 105 dependent on the \( a_w \) of the substrate.*

<table>
<thead>
<tr>
<th>( a_w ) (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-time (h)</td>
<td>3.0</td>
<td>nt</td>
<td>5.5</td>
<td>15.5</td>
<td>11.1</td>
<td>13.5</td>
<td>22.2</td>
<td>112</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-time (h)</td>
<td>9.4</td>
<td>nt</td>
<td>8.1</td>
<td>2.7</td>
<td>2.9</td>
<td>6.8</td>
<td>11.1</td>
<td>30.4</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>( \log N_{\text{max}} )</td>
<td>7.6</td>
<td>nt</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.3</td>
<td>7.3</td>
<td>6.1</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>nt</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>85.5</td>
<td>62.0</td>
<td></td>
</tr>
</tbody>
</table>

*nt = not tested; ng = not grown; ni = not inactivated.
TABLE 2c. Growth parameters (lag-time, mean generation time and log N_{max}) as well as inactivation value D of Z. rouxii strain LMZ 120 dependent on the a_{w} of the substrate.

<table>
<thead>
<tr>
<th>a_{w} (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-Time (h)</td>
<td>4.3</td>
<td>0.3</td>
<td>1.0</td>
<td>2.4</td>
<td>4.3</td>
<td>90.3</td>
<td>110</td>
<td>417</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-Time (h)</td>
<td>5.6</td>
<td>5.6</td>
<td>5.6</td>
<td>4.5</td>
<td>8.0</td>
<td>9.2</td>
<td>22.8</td>
<td>32.8</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Log N_{max}</td>
<td>7.2</td>
<td>7.0</td>
<td>7.0</td>
<td>6.9</td>
<td>6.9</td>
<td>7.4</td>
<td>7.3</td>
<td>6.7</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>278</td>
<td>455</td>
</tr>
</tbody>
</table>

*ng = not grown; ni = not inactivated.

TABLE 2d. Growth parameters (lag-time, mean generation time and log N_{max}) as well as inactivation value D of Z. rouxii strain LMZ 100 dependent on the a_{w} of the substrate.

<table>
<thead>
<tr>
<th>a_{w} (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-Time (h)</td>
<td>6.2</td>
<td>nt</td>
<td>2.1</td>
<td>1.6</td>
<td>2.0</td>
<td>152</td>
<td>343</td>
<td>312</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-Time (h)</td>
<td>2.6</td>
<td>nt</td>
<td>2.3</td>
<td>2.6</td>
<td>4.0</td>
<td>10.1</td>
<td>11.7</td>
<td>25.0</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Log N_{max}</td>
<td>7.2</td>
<td>nt</td>
<td>7.5</td>
<td>7.6</td>
<td>6.9</td>
<td>7.1</td>
<td>7.3</td>
<td>6.2</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>nt</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>105</td>
<td>99</td>
<td>99</td>
<td>74.4</td>
</tr>
</tbody>
</table>

*nt = not tested; ng = not grown; ni = not inactivated.

TABLE 2e. Growth parameters (lag-time, mean generation time and log N_{max}) as well as inactivation value D of Z. rouxii strain LMZ 111 dependent on the a_{w} of the substrate.

<table>
<thead>
<tr>
<th>a_{w} (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-Time (h)</td>
<td>10.6</td>
<td>11.9</td>
<td>13.1</td>
<td>12.5</td>
<td>12.4</td>
<td>25.8</td>
<td>113</td>
<td>130</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-time (h)</td>
<td>3.7</td>
<td>3.5</td>
<td>3.6</td>
<td>3.8</td>
<td>5.1</td>
<td>8.8</td>
<td>8.9</td>
<td>45</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Log N_{max}</td>
<td>7.7</td>
<td>7.8</td>
<td>7.6</td>
<td>7.5</td>
<td>7.3</td>
<td>7.5</td>
<td>7.4</td>
<td>6.5</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>267</td>
<td>262</td>
<td>220</td>
<td>200</td>
</tr>
</tbody>
</table>

*ng = not grown; ni = not inactivated.

TABLE 2f. Growth parameters (lag-time, mean generation time and log N_{max}) as well as inactivation value D of Z. rouxii strain LMZ 114 dependent on the a_{w} of the substrate.

<table>
<thead>
<tr>
<th>a_{w} (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-Time (h)</td>
<td>5.6</td>
<td>5.5</td>
<td>5.8</td>
<td>5.1</td>
<td>9.5</td>
<td>11.8</td>
<td>19.5</td>
<td>57.6</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-time (h)</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.7</td>
<td>3.3</td>
<td>6.3</td>
<td>11.3</td>
<td>31.7</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Log N_{max}</td>
<td>7.9</td>
<td>7.7</td>
<td>7.8</td>
<td>7.6</td>
<td>7.7</td>
<td>7.4</td>
<td>7.5</td>
<td>7.2</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>990</td>
<td>445</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ng = not grown; ni = not inactivated.

TABLE 2g. Growth parameters (lag-time, mean generation time and log N_{max}) as well as inactivation value D of Z. rouxii strain LMZ 127 dependent on the a_{w} of the substrate.

<table>
<thead>
<tr>
<th>a_{w} (30°C)</th>
<th>0.998</th>
<th>0.995</th>
<th>0.983</th>
<th>0.958</th>
<th>0.913</th>
<th>0.837</th>
<th>0.795</th>
<th>0.760</th>
<th>0.701</th>
<th>0.626</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-Time (h)</td>
<td>3.8</td>
<td>4.2</td>
<td>5.9</td>
<td>3.7</td>
<td>7.0</td>
<td>24.0</td>
<td>53.0</td>
<td>341</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>g-time (h)</td>
<td>2.9</td>
<td>3.0</td>
<td>3.0</td>
<td>3.1</td>
<td>4.0</td>
<td>11.4</td>
<td>17.8</td>
<td>46.1</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Log N_{max}</td>
<td>7.7</td>
<td>7.8</td>
<td>7.6</td>
<td>7.5</td>
<td>7.4</td>
<td>7.8</td>
<td>7.1</td>
<td>6.8</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>D-Value (h)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>2065</td>
<td>179</td>
<td></td>
</tr>
</tbody>
</table>

*ng = not grown; ni = not inactivated.

Type 3. Seventeen inactivation curves distinguished by a constant linear reduction in the CFU/ml counts of the cultures were obtained with Z. bailii strain LMZ 108 at a_{w} <0.837 and with all other strains at a_{w} 0.701 and a_{w} 0.626. In Fig. 4, inhibition curves depicting data from experiments on Z. bailii strain LMZ 108 are shown as examples for the type 3 inactivation curve.

By plotting the calculated g-values against the water activity, optimum a_{w} for growth was obtained. As reported in Fig. 5, Z. bailii strain LMZ 108 showed less tolerance than the other strains. Optimum a_{w} for its growth was slightly below 1.00 and decreased a_{w} caused a noticeable and rapid inactivation of the cells. As Fig. 6 illustrates, the lower the a_{w}, the faster the inactivation (small D-values). Z. rouxii strains LMZ 100, LMZ 111, LMZ 114 and LMZ 127 showed similar characteristics: optimum a_{w} for growth was in the range of 0.98 to 0.998. The growth at a_{w} >0.90 was identical for all
strains; nevertheless larger differences were noted at $a_w < 0.85$, where the growth responses of strains LMZ 114 and LMZ 100 (g-values at $a_w 0.760 = $approximately 30 h) were better than those of strains LMZ 111 and LMZ 127 (g-values at $a_w 0.760 = $approximately 55 h).

Special note must be made of Z. rouxii strains LMZ 105 and LMZ 120; optimum growth was obtained at approximately $a_w 0.96$ with strain LMZ 120 and in the $a_w$ range of 0.913-0.958 with strain LMZ 105. The latter did not grow better than others at $a_w 0.760$; however, according to its evident requirement of high sugar concentrations for optimum growth and particularly high generation times at high $a_w$, it seemed appropriate to redefine strain LMZ 105 as “osmophilic”. Strain LMZ 120 exhi-
bited a relative broad aw optimum compared with strain LMZ 105. In addition, the g-values at aw 0.998 did not differ greatly from that at aw 0.958. For this reason to designate LMZ 120 as “osmophilic” was not appropriate. A non-linear relationship between lag-times and the aw-value was observed. Often the adaptation phase at aw 0.958 was very short. The highest log Nmax were always noted at aw >0.99; a decrease in the aw caused a perceptible decrease of the maximum cell yield.

**DISCUSSION**

**Relationship between aw, optical density and cell volume**

In accordance with previous investigations (10,30), in the present work only cell size and thus cell volume alterations were observed. Scarr (30) noted that cells actively grown at low aw were “slightly” smaller. She affirmed, however, that on transfer to high concentrations of sucrose, 50-90% of the cells assumed a transient shrunken condition with dense cell contents and became half their normal size. These statements were confirmed later by Ingram (10) who made an unequivocal distinction between osmotic shock effect after transference and the effects of actively growing the cells at different aw. Hence a very important consequence of such size alterations is the inadequacy of comparing the growth of microorganisms at different water activity values by optical density measurements. Cell size alterations with respect to aw were well recognized, but unfortunately studies on the relationship between optical density (OD) and cell volume at different aw values were incomplete and necessitated further investigation.

Rose (29) investigated the relationship between OD and cell volume at different aw values, but his trials were done with cells exposed to osmotic shock and not with actively growing microorganisms. Other authors pointed out the relationship between OD and dry cell weight (11,20). In an investigation with osmotolerant yeasts, the calibration of turbidity curves against Dry Cell Weight (DCW) should be avoided, however, because the intracellular retention of high molecular compounds such as polyols, especially at low aw, might lead to erroneous optical as well as gravimetric results.

**Influence of aw on growth**

Results of previous studies on the aw-tolerance by osmotolerant yeasts are contradictory. Water activity tolerance limits in a range from aw 0.62 (33) to approximately aw 0.85 (31) were found with strains supposed to have osmotolerant properties. Such low tolerance limits as those recorded by von Schelhorn (33) have not been confirmed in later investigations. Conversely Mossel (21) demonstrated that a strain of Z. rouxii was capable of growing in 70% (w/w) fructose only (aw 0.760). Recca and Mrak (25) tested several osmotolerant yeast strains isolated from orange juice concentrates, but none could grow in solutions more concentrated than 65°Brix (aw 0.865 at 25°C). English (9) noted growth of a strain of Z. rouxii at 73% RH (aw 0.73). A strain of Z. rouxii tested by Anand and Brown (1) grew at aw 0.765, whilst several other experimenters could not observe growth of osmotolerant yeasts at aw <0.85 (15,16,28,31).

Disagreement within previous literature could be due to different experimental conditions, e.g. different solutes, different measuring devices and procedures, to preconditioning with respect to aw (24,45) as well as to slight genetic variations within the species (26). Moreover, although sugar concentration in growth or fermentation media is usually expressed as % w/w (13,21,28), as % w/v (1,23) or as °Brix (15), these expressions are likely to be confused. In discussion of solute tolerance of microorganisms, the aw of the media would be preferable to % solute. Therefore direct correlations among the data produced by previous researchers and this investigation may not be appropriate.

However, some generalisations can be made. In accordance with Anand and Brown (1) all the strains tested in the present work could not grow at aw <0.760 and exhibited relatively broad aw-optima. However Anand and Brown (1) did not find any strain showing a requirement for a reduced aw, although there was limited evidence that some might grow optimally under drier conditions than others. Their findings supported Onishi’s (24) doubts about the existence of obligately osmophilic organisms. In spite of these prior assertions, in the present investigation strain LMZ 105 was designated as osmophilic because of its optimal growth rate at low aw values (0.913-0.958) and weak growth just below aw 1.00.

Since the fundamental paper of von Richter (27) many yeast strains able to grow in high sugar or salt concentrations have been isolated. With few exceptions (12,23,42), none has shown an obligate requirement for low aw, as that shown in the present trials by Z. rouxii strain LMZ 105, except at elevated temperatures for certain strains (17,36). Onishi (24) postulated different tolerances in accordance with the origin of the strain. The results of the present trials could partially confirm his postulate. First of all, Z. bailii strain LMZ 108, which was freshly isolated from spoiled fitness drink of aw 0.913, could not grow at aw <0.837 and was the least tolerant strain of those studied. Second, since the yeasts have been pre-enriched in a broth of aw 0.963, preconditioning with respect to aw could explain why several strains showed fast adaptation at aw 0.958. Taking into account these earlier findings and results of the present investigations, it is believed that the sugar tolerance of osmotolerant yeasts differs considerably, strain by strain, and there can be no doubt that strains with different tolerances for different aw values may exist within the same species.

The present knowledge on the molecular basis of the osmotolerance in yeasts is largely due to the investigations of Brown and co-workers. The literature on this research field was reviewed by Brown (3,4) and by Spencer and Spencer (37).

Even if the interest in the osmotolerant yeasts is mainly due to their role as spoilage agent, they possess proper-
ties that may be put to beneficial and economical use. The microbial production of polyols by fermentation and production of traditional fermented foods, especially in the Far East, as miso-paste and soy sauce are two such uses (24,37,38,45). Moreover, large-scale baking experiments carried out by Windisch and Stechowski (44) resulted in products which suggested that special osmotolerant yeast strains could be a means to compensate for a chemical leaving agent with doughs rich in fat and sugar and poor in water.

REFERENCES