Cardinal Temperatures for Growth of Osmotolerant Yeasts in Broths at Different Water Activity Values

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ABSTRACT

All three cardinal temperatures (T_{min}, T_{opt} and T_{max}) for growth of 6 strains as well as T_{min} and T_{max} for growth of an additional 23 strains were determined in solutions of 10, 30, 50 and 60% (w/w) glucose at a_{w} (20°C) of 0.990, 0.970, 0.922 and 0.868, respectively. The T_{opt}, for growth of Zygossaccharomyces rouxii and Z. bisporus were 24-28.5°C at a_{w} >0.990 and 31-33°C at a_{w} in the range of 0.922-0.868. Z. bailii showed T_{opt} for growth of 29-31°C and 33-35°C at a_{w} >0.990 and a_{w} <0.922, respectively. The T_{opt} for growth of Torulaspora delbrueckii was 27-28.5°C at a_{w} <0.990 and 31-33.5 at a_{w} in the range of 0.922-0.868. Debaryomyces Hansenii showed a T_{opt} of 24°C and 27-29.5°C at a_{w} >0.990 and a_{w} <0.922, respectively. The T_{min} and T_{max} for growth were also shifted toward higher values as the a_{w} decreased; at a_{w}<0.922 none of the tested strains grew at 4°C within 30 d. Several strains could grow at 42°C only in the presence of high sugar concentrations.

Osmotolerant yeasts were reported to be similar to mesophilic yeasts in that they grew over the temperature range 0°C-40°C with an optimum temperature of approximately 27°C (18). It is, however, a general phenomenon, observed with bacteria, molds and yeasts, that the optimum temperature for growth is considerably higher in solutions of high salt or sugar concentration. Moreover, failure of certain strains to grow at low a_{w} was found to be reversible at certain incubation temperatures (13,16). Christian (3) found that the NaCl requirement for growth of Halobacterium Halobium was less at lower temperatures. Ingram (10) made similar observations with other species of salt-tolerant bacteria. Horner and Anagnostopoules (8) studied the effect of lowering the incubation temperature, coupled with reduced a_{w}, using the molds Aspergillus Niger, Penicillium spp. and Rhizopus spp.; the combined effect of both temperature and a_{w} decrease resulted in longer shelf lives than those predicted from single variable experiments.

Investigations with osmotolerant yeasts are seldom undertaken. An increased T_{opt} for the growth of Zygossaccharomyces rouxii in concentrated sugar broths was recorded (9,11,12), whilst some osmotolerant yeast strains including Z. rouxii were able to grow at high temperatures, such as 40°C, in saline media, but not in conventional media. At 40°C sugars at concentrations higher than 30% and salt at concentrations exceeding 4% were effective in supporting growth (13). Scarr and Rose (16) isolated 2 strains of Z. rouxii and Torulaspora kestoni, which grew at 37°C, only on osmophilic agar but not on media containing 2% sugar. Unfortunately, all these previous investigations were not complete; very often only single strains, especially of Z. rouxii, were tested over narrow ranges of temperature and sugar concentration. Temperatures below 15-17°C and sugar or salt substrates at a_{w}<0.90 were never investigated. The purpose of the present work was, therefore, to investigate the influence of the substrate-a_{w} on T_{min}, T_{opt} and T_{max} for growth of several osmotolerant yeast strains, belonging to 6 different species (Z. rouxii, Z. bailii, Z. bisporus, D. Hansenii, T. delbrueckii and S. cerevisiae). The growth was investigated at temperatures in the range 4-46°C at four different substrate-a_{w} values.

MATERIALS AND METHODS

Preparation of media and solutions

All media used in the present investigation, i.e. yeast extract glucose (YEG) 10 broth [a_{w}(20°C) 0.990; pH 4.5; 10% (w/w) D-glucose; pure, Bender and Hobein, Zürich; 0.5% (w/w) yeast extract, BBL No. 11929]; yeast extract glucose (YEG) 30 broth [a_{w}(20°C) 0.970; pH 4.5; 30% (w/w) D-glucose; 0.5% (w/w) yeast extract]; yeast extract glucose (YEG) 50 broth [a_{w}(20°C) 0.922; pH 4.5; 50% (w/w) D-glucose; 0.5% (w/w) yeast extract]; yeast extract glucose (YEG) 60 broth [a_{w}(20°C) 0.868; pH 4.5; 60% (w/w) D-glucose; 0.5% (w/w) yeast extract]; yeast extract glucose (YEG) 50 agar [a_{w}(30°C) 0.906; pH 4.05; 50% (w/w) D-glucose; 0.5% (w/w) yeast extract]; 1.5% (w/w) agar, Oxoid No. L13] as well as the diluent DS30 [a_{w}(30°C) 0.958; pH 4.75; 30% (w/w) D-glucose; 0.85 (w/w) NaCl, 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.

Physical methods

\( a_w \) Measurement. The \( a_w \) of the yeast extract glucose broths was taken at 4, 10, 20, 30, and 42°C, with a hygrometer model \( a_w \)-Box with measuring station EEJA, supplied by Novasina AG, Zürich. The equipment was calibrated at 20°C using Equilibrium Relative Humidity (ERH) values (%) of standard saturated salt solutions (7). The \( a_w \) of YEG50 agar and diluent DS30 was taken at 30°C only, using the same equipment.

pH-Adjustment. All YEG-broths were adjusted to pH 4.5 with a few drops of 85% orthophosphoric acid (Merck No. 573).

Microbiological methods

Preenrichment and preparation of inocula. YEG30 broth was inoculated from stock cultures on agar slants and incubated at 30°C for 60 h. The yeast was then harvested by centrifugation (Heraeus Centrifuge, Osterode, GFR; Relative Centrifugal Field (RCF) 2060 x g; 5 min) and resuspended in diluent DS30. Inoculation suspensions (\( 10^5 \) cells/ml) were prepared by counting with a Hefler counting chamber and appropriate dilution with DS30.

Incubation of cultures. The cultures were incubated in Temperature Gradient Incubators (TGI) or in conventional laboratory incubators.

Trials with Temperature Gradient Incubators (TGI)

Yeast taxa. Six strains: Zygasscharomyces rouxii strain LMZ 105, Z. bailii strain LMZ 108, Torulaspora delbrueckii strain LMZ 1901, Debaryomyces Hansenii strain LMZ 1902 (all previously isolated from various spoiled high-sugar foods) as well as Z. bisporus strains CBS 702 and Saccharomyces cerevisiae strain CCB 7809 were used in this investigation.

Description of the equipment. The Temperature Gradient Incubator (Scientific Industries Ltd., New York; supplied by Bender and Hobein, Zürich) is a multi-temperature, multi-sample unit, which allows the simultaneous incubation of 60 samples at 30 different temperatures (2 samples at each gradient level) in a single self-contained precision instrument. The liquid samples can be incubated in glass L-tubes, optically selected to permit spectrophotometric measurements without sample transfer. A built-in oscillator provides mixing and aeration of the samples in the tubes. Thirty temperatures, spaced along the temperature gradient block, can be tested. Any two gradient end-point temperatures as well as the frequency of oscillation can be selected at the front panel dials.

Selection of gradient end-points. Two TGI placed in a room at constant temperature of 22 ± 0.5°C were used: for both TGI temperatures of +4°C and +46°C were selected as lower and upper gradient end-points; four temperature gradients were therefore established.

Selection of frequency of oscillation. To provide sufficient mixing but no foaming of the cultures a frequency of 20 oscillations/min was selected.

Recording exact temperatures. For each TGI exact determination of the 30 temperatures was carried out in a preliminary series of trials, by placing calibrated "Thermocoax" miniature-coated thermocouples (chrom-alumel couple coated with stainless steel; diameter = 0.1 mm; manufactured by Philips AG Zürich) in L-tubes filled with distilled water and equilibrated at each gradient level. The temperatures at each level were monitored and recorded at 24-s intervals using a multi-point recorder (Transokomp 250, Philips AG). The accuracy of each gradient level was within the range ±0.3°C. Moreover, at regular intervals (12 h) during the investigation the constancy of the selected end-point temperatures was checked using the same thermocouples.

Determination of minimum, optimum and maximum temperature for growth. For each sugar concentration [10, 30, 50 and 60% (w/w) D-glucose] 30 L-tubes each containing 15 ml of broth were sterilized by autoclaving at 110°C for 15 min. After equilibration at the desired temperatures, all broths were inoculated each with 0.1 ml suspension to achieve an initial yeast count of \( 6.6 \times 10^2 \) cells/ml. The initial optical density (OD) was recorded by inserting each culture tube directly in a spectrophotometer (Perkin/Elmer model Coleman 44). The cultures were then incubated along the T-gradient each at a different temperature for 30 d. The OD was recorded at regular intervals (2-8 h) as described above. The temperature, at which an increase of 0.1 OD was first recorded, corresponded to the \( T_{op} \) for growth. If growth was observed simultaneously at two or more temperatures, an optimum temperature range occurred. \( T_{min} \) and \( T_{max} \) for growth were the lowest and the highest temperatures at which growth occurred within 30 d of incubation.

Trials with conventional laboratory incubators

Yeast taxa. Twenty-three strains: Zygasscharomyces rouxii strains LMZ 100, LMZ 102, LMZ 104, LMZ 106, LMZ 107, LMZ 110, LMZ 111, LMZ 112, LMZ 114, LMZ 115, LMZ 116, LMZ 117, LMZ 118, LMZ 119, LMZ 120, LMZ 122, LMZ 126, LMZ 129, LMZ 130 and Z. bailii strain LMZ 109, all isolated from various spoiled high-sugar products as well as Z. rouxii strains CBS 732 and CBS 736 and Torulaspora delbrueckii strain CBS 1090 were used in this investigation.

Selection of incubation temperatures. To assess the \( T_{min} \) for growth, cultures were incubated at 4, 6.5, 10 and 15°C in refrigerated cabinets without forced air circulation. Incubator cabinets with forced air circulation at 34, 37, 40, 42 and 46°C were used to investigate the \( T_{max} \) for growth.

Recording exact temperatures. The temperatures of each of the refrigerators and the cabinets was measured by placing a thermocouple in a control tube containing deionized water. The temperature of each incubator was monitored and recorded at 12-min intervals during the entire experiment. The temperature constancy in the refrigerators and cabinets was ±0.5°C and ±0.2°C, respectively.

Determination of minimum and maximum temperature for growth. With each of the 23 strains tested, 4 substrates (YEG10, YEG50, YEG50 and YEG60 broths) were incubated for 30 d at each of the 9 tested temperatures. An initial count of \( 10^8 \) cells/ml was attained by inoculating 9.9 ml of sterile substrate in reagent tubes with 0.1 ml of inoculum. Twice a day the culture tubes were shaken and presence or absence of growth was determined by placing them against a white card bearing lines, drawn with black India ink, approx. 3/4 mm wide. If growth in the tubes completely obliterated the lines or if the lines appeared as diffuse bands or with indistinct edges the reaction was considered as to be "positive." \( T_{min} \) and \( T_{max} \) for growth were the lowest and the highest temperatures at which growth was observed within 30 d of incubation.

RESULTS

Cardinal temperatures for growth determined with TGI

Because of the large number of curves required to illustrate all the data collected, only curves connected with experiments carried out with Z. rouxii LMZ 105 in
YEG30 and YEG60 are shown. These are presented in Fig. 1 sections A and B, respectively. The growth behavior of Z. rouxii LMZ 105 in YEG30 and YEG60 are presented, because an optimum temperature range (25-26.5°C) and an optimum temperature peak (33.5°C), respectively, were observed. The results of the remaining strains are summarized in Fig. 2. Figure 1 and Fig. 2 show that an increased sugar concentration in the substrate caused higher cardinal temperatures for growth of all the strains tested.

Minimum temperature for growth; $T_{\text{min}}$. An increase in the glucose concentration of the substrate from 10 to 60% caused an increase of $T_{\text{min}}$ by approx. 5.6°C on average. All strains but Z. bailii LMZ 108 grew at 4°C within 30 d in the presence of 10% glucose. Only 3 strains could grow at 4°C in a substrate with 30% glucose. None of the tested strains was able to grow within 30 d at 4°C when the glucose concentration in the substrate was 50% or higher; at these glucose levels the $T_{\text{min}}$ was in the ranges of 12-13°C for Z. bailii LMZ 108 and 6.5-10°C for the other strains tested.

Optimum temperature for growth; $T_{\text{opt}}$. An increase in the sugar concentration of the substrate from 10 to 60% increased the $T_{\text{opt}}$ by approx. 6°C on average. At low glucose concentrations (10%) Z. bailii LMZ 108 showed a $T_{\text{opt}}$ in the range 29-31°C. The $T_{\text{opt}}$ of the remaining strains ranged from 24°C (D. hansenii LMZ 1902) to 28.5°C (Z. bisporus CBS 702). At higher glucose concentrations (60%) Z. bailii LMZ 108 showed a $T_{\text{opt}}$ in the range of 33.5-35°C; $T_{\text{opt}}$ for the strains ranged from 27-29.5°C (D. hansenii LMZ 1902) to 33.5°C (Z. rouxii LMZ 105, T. delbrueckii LMZ 1901 and Z. bisporus CBS 702). Broad optimum temperature ranges were exhibited by 3 strains in YEG60, 4 strains in YEG50 and 1 strain each in YEG30 and YEG10.

Maximum temperature for growth; $T_{\text{max}}$. An increase in the glucose concentration of the substrate from 10 to 60% raised $T_{\text{max}}$ by approx. 4.4°C on average. Z. rouxii strain LMZ 105 was the only strain that could grow at 37°C at each glucose level tested, whilst T. delbrueckii LMZ 1901 and Z. bisporus CBS 702 grew at 37°C only in the presence of at least 30% glucose. D. hansenii LMZ 1902 could grow at 37°C only in 60% glucose; $T_{\text{max}}$ for its growth was 32°C and 37°C in YEG10 and YEG60, respectively. Z. rouxii LMZ 105 showed higher $T_{\text{max}}$ in the range 37-42°C, dependent on the glucose level in the substrate.

Special note must be made of Saccharomyces cerevisiae CCB 7809. This strain was not able to grow in 60% (w/w) glucose. At lower temperatures it showed the same behavior as the other osmotolerant strains tested. The increase of $T_{\text{opt}}$ and $T_{\text{max}}$ at increased sugar levels was, however, very slight; $T_{\text{opt}}$ and $T_{\text{max}}$ increased by 3°C and 1.5°C, respectively, when the glucose level in the substrate increased from 10 to 50%.

Minimum and maximum temperature for growth determined in conventional laboratory incubators (Table 1)

Minimum temperature for growth; $T_{\text{min}}$. At 4°C none
of the tested strains grew in YEG50 or YEG60; a decrease in the glucose concentration, e.g. 10%, allowed 14 strains to grow. A temperature of 6.5°C was sufficient to inhibit growth of 3 strains (Z. rouxii LMZ 104, LMZ 116, and LMZ 129) at any sugar level; at this temperature in YEG50 and YEG60 no growth was observed with 5 and 15 strains, respectively. All strains grew at 10°C, if the glucose concentration did not exceed 30%. At increased sugar concentration, i.e. 50% and 60%, 2 and 3 strains respectively failed to grow. All strains tested grew at 15°C regardless of the glucose concentration in the growing broth. T. delbrueckii CBS 1090 did not grow in 60% w/w glucose. In general, the higher the sugar concentration in the substrate, the higher the minimum temperature for growth.

**Maximum temperature for growth:** \( T_{\text{max}} \). All tested strains grew at 34°C. However, 34°C was the \( T_{\text{max}} \) for growth of Z. rouxii LMZ 104 in low concentrated glucose broth (10% glucose). At this concentration all other 22 strains exhibited a \( T_{\text{max}} \) of 37°C. In YEG30 18 strains showed a \( T_{\text{max}} \) of 37°C; Z. rouxii LMZ 112 was able to grow up to 40°C, whilst for 4 others (Z. rouxii strains LMZ 100, LMZ 102, LMZ 116, and LMZ 130) growth was inhibited at temperatures higher than 42°C. In YEG50 37°C was the \( T_{\text{max}} \) for the growth of 12 strains, 40°C the \( T_{\text{max}} \) for 4 strains and 42°C for the remaining 7 strains. In concentrated sugar broth (60% glucose) all strains tolerated 40°C; 9 strains were even able to grow at 42°C. However, Z. rouxii strains LMZ 104 and LMZ 120 did not grow at temperatures higher than 37°C. At this concentration T. delbrueckii CBS 1090 did not grow within 30 days. In general, the 23 osmotolerant yeasts tested showed a maximum temperature for growth of 37°C. However, with several strains the maximum temperature for growth could be increased to 42°C by increasing the sugar concentration in the growing broth.

**DISCUSSION**

The culture broths used in the present work have been defined in terms of glucose concentration. Problems arise if the hydration of these broths is expressed as water activity (a\(_w\)). In a separate series of trials (results not shown), a\(_w\)-measurements of the culture broths at 5 different temperatures resulted in a slightly negative dependence of a\(_w\) on temperature. Therefore, although the glucose concentration (% w/w) did not vary over the tested temperature range, the a\(_w\) of the broths was different at each incubation temperature. Nevertheless, because of the negative characteristic of the a\(_w\)-temperature relationship,

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**TABLE 1. Minimum and maximum temperatures for growth of 23 osmotolerant yeast strains at various levels of glucose during incubation for up to 30 days.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>( T_{\text{min}} ) (°C) for growth</th>
<th>( T_{\text{max}} ) (°C) for growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose level (%w/w)</td>
<td>10</td>
<td>30</td>
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<tr>
<td>Own isolates</td>
<td></td>
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</tr>
<tr>
<td>Z. rouxii</td>
<td></td>
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<tr>
<td>LMZ 100</td>
<td>≤4</td>
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<tr>
<td>LMZ 102</td>
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<td>LMZ 116</td>
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<td>LMZ 129</td>
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<td>LMZ 111</td>
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<td>LMZ 118</td>
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<td>LMZ 110</td>
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<td>LMZ 114</td>
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<td>LMZ 119</td>
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<tr>
<td>LMZ 112</td>
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<td>LMZ 104</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Z. bailii</td>
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<tr>
<td>LMZ 109</td>
<td>6.5</td>
<td>6.5</td>
</tr>
</tbody>
</table>

| Reference strains |     |     |     |     |     |     |     |     |
| Z. rouxii         |     |     |     |     |     |     |     |     |
| CBS 732           | ≤4  | ≤4  | 6.5 | 6.5 | 37  | 37  | 37  | 40  |
| CBS 736           | 10  | 10  | 15  | 15  | 37  | 37  | 37  | 40  |
| T. delbrueckii    |     |     |     |     |     |     |     |     |
| CBS 1090          | ≤4  | ≤4  | 15  | --  | 37  | 37  | 40  | --  |
absence of growth at low temperatures was due to tempera-
ture only and not to supplementary a_w-inhibition. On
the other hand, in the upper temperature range lower a_w
than predicted could have delayed the appearance of
growth. However, these a_w-values were never so low as
to completely inhibit the growth of the tested osmotoler-
ant yeasts. Since the alterations of the a_w over a narrow
temperature range (3-4°C) are very small (0.003-0.004
a_w) the determination of T_{opt} for growth has not been
influenced. Concluding, temperature-induced variations of
a_w within the tested temperature range did not affect the
determination of the cardinal temperatures for the growth of
osmotolerant yeasts.

In the present work, the a_w has been controlled using
glucose as humectant. However, earlier investigators
suggested that the type of humectant does not play any
role in the interaction of growth temperature and osmotic
conditions of substrate (8,9,13-15).

The information available at present does not allow
formulation of a general rule for the relationship between
temperature and growth of osmotolerant yeasts dependent
on the concentration (sugar or salt) of the substrate. Most
of the previous investigations were undertaken testing
single strains of Z. rouxii at temperatures in the upper
range of their growth only (4,11,13,14,16). For example
Ingram (11) investigated growth of Z. rouxii in the T-
range of 15-40°C, whilst temperatures within the ranges
of 17-37°C and 22-40°C were adopted by English (4) and
Restaino et al. (14), respectively. Nevertheless, previous
studies have revealed, with a constant general agreement,
that the power of growth or even survival of Z. rouxii
over the range of 35-37°C is greatly enhanced by addition
of small amounts of sugar or salt to the medium (4,9,11-
14,16). Moreover, addition of sugar to the substrate in-
creased the T_{opt} for growth (11,14).

In the present work, growth of 29 osmotolerant strains
belonging to 6 different species was investigated over the
temperature range of 4.46°C, at four different glucose
levels. As a result, osmotolerant yeasts showed higher
cardinal temperatures (T_{min}, T_{opt}, T_{max}) at an increased
solute concentration in the substrate. Previous statements
obtained with strains of the species Z. rouxii can there-
fore be generalized to all osmotolerant yeasts, since only
slight differences were noted among the six tested os-
motolerant species.

Two important practical consequences of the results of
the present investigation on T_{min} are: (a) the knowledge
that benefits could derive from storing intermediate mois-
ture foods (IMF) or their raw ingredients, of approxi-
mately a_w 0.85 or lower, at chilling temperatures. Stor-
age of IMF-raw ingredients at temperatures of approxi-
mately 4°C should provide protection against growth of
osmotolerant yeasts. This holds true also for final IMFs
whose packaging has already been opened, such as jam,
ungels, canned fruit concentrates, etc. (to inhibit growth
of possible recontaminants); (b) an incubation temperature
of 32°C, instead of the traditional 25 to 30°C as adopted
for osmotolerant yeasts, would be more efficient for de-
tection, isolation and enumeration purposes in the pres-
ence of high sugar concentrations.

As suggested by Restaino et al. (14), the protective
effect of intracellular solutes on the metabolic
mechanisms could explain the increased T-values when the
sugar concentration in the growing broth increased.
Polyols (arabitol, mannitol, glycerol, etc.) are retained in
osmotolerant yeast cells growing at low a_w. They func-
tion as “osmoregulators” equilibrating the outside and
inside osmotic pressure through intracellular bound water
and protect enzymes against inhibition and/or inactivation
(1,2). Although the mechanisms of protection towards en-
zymes had not been elucidated, Restaino et al. (14)
suggested that this system could be related to heat-sensi-
tive enzymes with respect to elevated temperatures.

In their experiments, Spencer et al. (17) indicated that
production of polyols by osmotolerant yeasts was in-
creased considerably by raising the temperature from 30
to 37°C. Therefore, since the lower the a_w, the more
polyols are retained within the cells, the overall protec-
tive action is increased at increased temperatures and re-
duced a_w of the culture broth.

However, temperature does not interact with a_w only;
other environmental factors have already been shown to
play an important role (5,6). T_{min}, T_{opt}, and T_{max}
reported are valid only under specific conditions of time,
medium and method of measurement. Although no infor-
mation is available on osmotolerant yeasts, the nutritional
requirement of some microorganisms varies during
growth at temperatures above T_{opt}. A strain of Coprinus
fimetarius, which had a T_{opt} between 35 and 40°C, was
able to grow up to 44°C only if the growth medium was
supplemented with methionine or hemocystine (5).
Biotin was essential for growth of Aspergillus niger in
a rhamnose medium at high temperature (6).

In addition to the factors already mentioned, many
others could be expected to affect the temperature re-
sponse of microorganisms. Therefore, predictions based
on laboratory data might be invalidated by nutritional dif-
cences between the natural products and the artificial
media.

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   Aspergillus niger when grown on a rhamnose medium at high tem-
   fects of water activity, pH and temperature on the growth and


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**Jermini et al., con't. from p. 472**


