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Evaluation of injury to *Saccharomyces rouxii* YSa40 cells in low water activity/pH glycerol/CPB stress system

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Abstract: Mid-exponential phase *Saccharomyces rouxii* YSa40 cells subsequently stressed at low a_w/pH in the 0.64 a_w/pH 3.5 glycerol/CPB system became injured. Such injury was detected by the loss of ability of the stressed population to form colonies on secondary-stress plating medium (glycerol/BM agar at 0.94 a_w/pH 3.5 (lactic acid)) while colony forming ability on secondary non-stress plating medium (sugars/BM agar at 0.94 a_w/pH 3.5 (lactic) was unaffected. The injury was shown to be due to sensitivity to glycerol/lactic acid. Results of the present study will be useful for achieving complete decontamination of 'Intermediate Moisture Foods' against xerotolerant molds and yeast.

Keywords: Decontamination, water activity, intermediate moisture foods, injury, physical treatments

Introduction

Generally, food processing treatments such as heat, irradiation, chemicals, freezing, freezing and thawing and freeze drying are routinely employed for an effective decontamination of a food product (Gould, 1996). However, if the treatments are inadequate, then the anticipated sterility might not be achieved and the surviving microbes may be stressed and injured (Hurst, 1980).

Generally, most of the foods are processed by the removal of available water to inactivate or kill the microbial population. The most ancient method of food preservation in this category was probably drying. However, in recent years, impetus has been given to the development of semi-moist foods (Chirife and Buera, 1995) which could be intended for military and space rations. This novel food is generally referred to as 'Intermediate Moisture Food' (IMF). IMF is characterized by a moisture content of between 15 to 50% and water activity (a_w) from 0.60 to 0.90. It has been reported that a reduction in water activity levels to be an effective preservation method of perishable food as growth of many of the spoilage microorganisms is retarded due to low water activity. IMF has become highly popular as these are shelf stable, retain nutritional and sensory qualities, incur in less transportation and storage costs (Karel, 1973; Leistner, 1992; Wang and Leistner, 1994; Thakur and Singh, 1995; Huang *et al*., 2010).

It is a generally accepted fact that even in low pH fruit-based IMF, xerophilic molds are potentially capable of growth and can be present as a spoilage hazard. It has been reported that *Saccharomyces rouxii* to have a remarkable ability to resist low a_w and grow down to a_w 0.62 (Restaino *et al*., 1983; El Halouat and Debevere, 1996). *Saccharomyces rouxii* is one of the common osmotolerant spoilage yeast that can even tolerate up to 22% salt (NaCl) and 80% glucose or sucrose concentrations in the culture medium (Onishi, 1963). One of the major problems that arises in the manufacture and marketing of low a_w fruit-based IMF with respect to this xerotolerant yeasts is the probability and significance of their subsequent increase or survival, if other environmental factors are favorable. Generally, xerotolerant yeasts present in substrates can remain viable for several weeks even in concentrated preservative solutions.

On prolonged environmental stresses at reduced water activity levels within the IMF range, surviving cells could be sub-lethally injured. Reports are available, wherein cells exposed to various environmental stresses like: heat treatment, irradiation addition of antimicrobial chemicals, freezing and thawing, freeze-drying and moisture reduction including dehydration have survived (Hurst *et al*., 1973; Chawla and Chander 2004; Wu *et al*., 2001; Wu, 2008).

Keeping this as background, the major objective of the present study was to demonstrate injury of low a_w glycerol stressed *Saccharomyces rouxii* YSa40 cells. Hence, accordingly, experiments were designed using a model of low a_w/pH stress system and subsequently paired plating media, bearing in mind that the possibility of using a medium containing 'Rose Bengal', low pH or high glycerol contents might be useful to manifest the extent of low a_w injury.

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Materials and Methods

Source and maintenance of organism

The organism used in the present study was a xerotolerant strain of *Saccharomyces rouxii* YSa40. This organism was kindly provided by Dr. R.H. Tilbury from Tate and Lyle Limited, Group Research and Developmental, Reading, England and the culture was identified by conventional diagnostic methods (Lodder, 1970). Further, the stock cultures were maintained on slants of Scarr's Osmophilic Agar (SOA) (Scarr, 1959) and stored in screw capped vials at 4°C. Stocks were sub-cultured at monthly intervals while working culture was obtained by streaking the cells onto SOA and incubating at 30°C for 2 days.

Preparation of basal medium and buffer solution

For the preparation of basal medium (BM) the method described by Anand and Brown (1968) was adapted with some minor modifications. The medium comprised of peptone (5.0 g), yeast extract (2.5 g), KH_2PO_4 (1.0 g), $MgSO_4.7H_2O$ (0.25 g), $CaCl_2$ (0.1 g), glucose (2 g) and distilled water (1 litre). The basal salts and glucose were made up as separate stock solutions at one hundred times their use concentration. These stock solutions were sterilized separately, stored in 100 ml quantities at 4°C and added aseptically to the separately sterilized (autoclaved at 115°C/10 min).

When citrate phosphate buffer (CPB) was added to adjust pH values of growth medium or glycerol systems, 20 ml of the water component of the medium or systems were replaced by 20 ml CPB solution mixtures containing appropriate different volumes of 0.1 M- citric acid and 0.2 M- dibasic sodium phosphate. The selected aw values were obtained by reference to the previously determined glycerol/water sorption isotherm using Sina-scope (Novasine AG, Zurich, Switzerland). The effect on aw of the substitution of water component by CPB solution mixtures and/or the addition of basal medium to the medium or systems could be ignored when checked with the Sina-scope. The pH values of the medium or systems were determined by a pH electrode meter (Electronic Instrument Limited, Model 7020). Medium or systems were autoclaved at 115°C/10 min; any weight loss was compensated by aseptic addition of sterile distilled water. This was used for both broth and agar media.

Inoculum and stressing procedures

A 3 day streak culture of *Saccharomyces rouxii* YSa40 (one loopful) grown on SOA was inoculated into 40 ml of growth medium, 0.94 a_w/pH 4.8 glycerol/CPB/BM in a 250 ml Erlenmeyer flask and incubated at 30°C in an orbital shaker (at 250 rpm/min.). The cells were grown for 24 h. Further, 0.03 ml of the 24 h culture was inoculated into 40 ml fresh growth medium in a 250 ml Erlenmeyer flask, giving an initial cell density of 4.0×10^6 cfu/ml in the medium. The cells were incubated under the same growth condition as described above for 10 h to reach the mid-exponential phase.

Mid-exponential phase, 0.94 a_w/pH 4.8 glycerol/CPB/BM grown cells were harvested by centrifugation at 3487 x g (Mistral 6L, centrifuge, M.S.E.) for 20 min at 30°C. The supernatant was discarded and the cell pellets were resuspended in 40 ml stress system (0.64 a_w/pH 3.5 glycerol/CPB) and 40 ml non-stress control system (0.94 a_w/pH 3.5 glycerol/CPB) both contained in sterile 250 ml Erlenmeyer flasks. Both stress or non-stress systems were prepared with various initial cell densities (as indicated in Table 1) and incubated in an orbital shaker (250 rpm/min) at 30°C. At various intervals as indicated in the table, aliquots from the systems were removed for sampling.

Enumeration

For viable counts of cells from stress and non-stress systems, diluents identical to the stress and non-stress systems were used respectively. Plating agars: Malt Extract Agar (MEA) + glycerol, MEA+ NaCl, and MEA+ sugars (sucrose and glucose mixture) were used at a wide range of aw values. Other modified basal medium plating agars: sugars (sucrose and glucose mixture)/BM agar, glycerol/BM agar, sucrose/BM agar and glucose/BM agar were also used at an equivalent 0.94 a_w/pH 3.5 with pH adjusted either with lactic acid or with citrate phosphate buffer.

In certain cases, where low a_w plating agars containing high solute concentrations were used, inoculated plates for incubation were stored at 30°C within desiccators over saturated salt solutions corresponding to the same a_w of the agars. The saturated salt solutions used were cupric chloride, 0.64 a_w; sodium chloride, 0.75; potassium chromate, 0.86 a_w and barium chloride, 0.89 a_w. These solutions maintained the proper aw of the agars and prevented drying of the agars. The time of incubation varied with aw and nature of solute of the plating agars.

Results

Table 1 depicts the results on the effects of composition of plating media on the recovery of stressed and non-stressed *Saccharomyces rouxii* YSa40 cells. Viable cell counts on 0.94 a_w/plating

media showed that incorporation of 0.003% w/v Rose Bengal into SOA at pH 5.4 did not significantly lower viable counts of the stressed cell population. Likewise, alteration of pH values on SOA to pH 3.5 or 7.3 did not seem to affect viable counts of stressed cells compared to SOA counts at pH 5.4. Hence the use of the above plating media permitted a high recovery of total survivors.

Table 1. The effect of composition of plating media on the recovery of stressed and non-stressed *Saccharomyces rouxii* YSa40 cells at 30°C

Enumeration agar media			Viable counts (cfu/ml)	
Medium	**Water activity (aw)**	**pH**	**Stressed Cells**	**Non-stressed cells**
SOA	0.94	5.4	2.47×10^5	1.95×10^7
SOA + 0.003% (w/v) rose bengal	0.94	5.4	2.00×10^5	1.79×10^7
SOA	0.94	3.5 (LA)	3.11×10^5	1.94×10^7
SOA	0.94	7.3 (NaOH)	2.25×10^5	2.30×10^7
MEA + 7% NaCl	0.96	5.4	5.37×10^4	2.11×10^7
MEA + 10% NaCl	0.94	5.2	5.40×10^4	1.41×10^7
MEA + 15% NaCl	0.90	4.9	5.59×10^4	1.60×10^7
MEA + 20% NaCl	0.84	4.6	NDG	NDG
MEA + 35% sucrose	0.96	3.5 (LA)	3.94×10^5	1.97×10^7
MEA + 35% sucrose+ 10% glucose	0.94	3.5 (LA)	3.33×10^5	2.14×10^7
MEA + 35% sucrose + 15% glucose	0.92	3.5 (LA)	2.75×10^5	2.01×10^7
MEA + 35% sucrose + 20% glucose	0.84	3.5 (LA)	1.72×10^5 *	1.98×10^7 *
MEA + 35% sucrose + 30% glucose	0.79	3.5 (LA)	1.65×10^5 π	1.85×10^7 π
MEA + 22.5% glycerol	0.94	3.5 (LA)	1.92×10^3	1.98×10^7
MEA + 40% glycerol	0.87	3.5 (LA)	3.58×10^3	1.75×10^7
MEA + 55% glycerol	0.77	3.5 (LA)	NDG	NDG
MEA + 66% glycerol	0.67	3.5 (LA)	NDG	NDG
MEA + 70% glycerol	0.62	3.5 (LA)	NDG	NDG
MEA + 35% sucrose + 10% glucose	0.94	3.5 (LA)	3.33×10^5	2.14×10^7
MEA + 10% NaCl	0.94	3.5 (LA)	1.11×10^4	2.08×10^7
MEA + 22.5% glycerol	0.94	3.5 (LA)	1.92×10^3	1.98×10^7

MEA= Malt Extract Agar;
SOA= Scarr's Osmophilic Agar (MEA plus 35% (w/w) sucrose, 10% w/w glucose and 55% w/w Water);
NDG= no detectable growth after 1 month incubation at 30°C;
LA=lactic acid;
* =1 week incubation at 30°C;
π=2 week incubation at 30°C

When the plating media: NaCl/MEA at 0.96-0.90 a_w/pH 5.4-4.9 were used to recover low a_w stressed cells, there was approximately five-fold reduction compared to plating on SOA controls at 0.94 a_w/pH 5.4. This indicated that low a_w stressed cells did become salt sensitive i.e., injury was demonstrated. However, the extent of this injury was considered insufficient as a model system for subsequent repair studies. Subsequently, no further increase in salt sensitivity was demonstrated even when the stress system was incubated for an extended 12 days period (data not shown). Table 1 also shows that no viable cell count was detected on NaCl/MEA at 0.84 aw/pH 4.6 for the stressed cells. Non-stressed, control cells, however, were not affected by all the above plating media mentioned earlier except with NaCl/MEA at 0.84 a_w/pH 4.6 where no detectable growth was observed.

Table 1 also shows the a_w of the sugars/MEA and glycerol/MEA obtained by varying the concentrations of solutes in MEA. It was observed that plate counts on glycerol/MEA at 0.87-0.94 a_w/pH 3.5 (lactic acid) values gave approximately 2-log cycle reductions in viability compared to plate counts on sugars (sucrose and glucose mixture)/MEA at 0.79-0.96 a_w/pH 3.5 (lactic acid) values. This demonstrated that the development of injury of low a_w glycerol stressed cells was based on the extreme sensitivity of stressed cells to glycerol/MEA but not to sugars/MEA. Non-stressed control cells, however, were not affected by the above plating media. It was noted for both stressed and non-stressed cells that visible colonies finally appeared on sugars (sucrose and glucose mixture)/MEA at 0.84 and 0.79 a_w after 1 and 2 weeks incubation respectively at 30°C. However, there was no detectable growth at 0.77-0.62 a_w/pH 3.5 (lactic acid) glycerol/MEA, even after one month of incubation (at 30°C) indicating an inhibitory effect at these aw values.

Results from Table 1 also show that when the agars were adjusted to the 0.94 a_w/pH 3.5 (solute/lactic acid) values for the recovery of low aw stressed cells, ten-fold reductions in viable counts were observed with the three plating media in the following decreasing order: sugars (sucrose and glucose mixture)/MEA>NaCl/MEA>glycerol/MEA. Hence the results confirmed that low aw glycerol stressed cells were indeed very sensitive to the glycerol/MEA agar. Non-stressed cells, however, were not affected by all these plating media.

In another attempt to optimize the plating agars for demonstration of injury, the composition of enumerating agars was modified by using basal medium plus 2% (w/w) bacto-agar as a substitute for MEA (since basal medium was used throughout for growth media preparation). To confirm that low a_w injury is caused by the sensitivity of cells to both glycerol and lactic acid, various enumerating agars comprising of sugars (alone or in combination)/BM agars and glycerol/BM agars adjusted with lactic acid or citrate phosphate buffer (CPB) were prepared at 0.94 a_w/pH 3.5. It was also observed that low $a_w$stressed cells were very sensitive to lactic acid in the glycerol/BM agar at 0.94 a_w/pH 3.5 giving 2-log cycle reduction in viability compared to plate counts on the sugars (sucrose and glucose mixture)/BM agar at 0.94 a_w/pH 3.5. However, CPB in the glycerol/BM agar gave only a 64% reduction in viable count compared to the sugars (sucrose and glucose mixture)/BM agar counts at 0.94 a_w/pH 3.5 (lactic acid) and this was considered insufficient as a paired plating media system for demonstration of injury. Plating media composed of individual sugar (sucrose

or glucose)/BM gave a similar high recovery of low aw stressed cells as the sugars (sucrose and glucose mixture)/BM agars at the same 0.94 a_w/pH 3.5 (lactic acid or CPB). Cells in the non-stress control system were unaffected by all the plating media used.

Discussion

The protective effects of sugars versus the toxic effects of other solutes such as NaCl and glycerol in MEA on the recovery of low aw stressed cells was demonstrated over a wide range of solutes concentrations or at the same 0.94 a_w/pH 3.5 (lactic acid) (Table 1). Also, low aw stressed cells plated onto sugars (alone or in combination)/BM agars were approximately 2-log cycles higher in viability than those plated onto glycerol/BM agar at the same 0.94 a_w/pH 3.5 (lactic acid). Whilst injured cells were sensitive to glycerol/lactic acid in the glycerol/BM agar, it seemed that injured cells could probably repair low aw injury on sugars/BM agar containing sugars/lactic acid.

Several theories have been put forward to account for the protective effects of sugars. Earlier, Choate and Alexander (1967) have explained that sugar which cannot readily permeate the cell membrane could act as an 'osmotic buffer', hence protecting cell constituents (membrane, protein, nucleic acid, etc.). It is interesting to note that agars containing high levels (30 to 50% w/w) of sugars (i.e., sucrose, glucose, fructose) have been routinely used as selective media for the isolation of xerotolerant yeasts including *Saccharomyces rouxii* from substrates with high solute level usually sugar (Pitt, 1975; Tilbury, 1980; Beuchat, 1981; Elgadi *et al*., 2008). Assuming that a proportion of cells had been injured, injured cells could probably repair their damage on this selective media giving maximal numbers of cells in surviving population (Table 1).

In one of our preliminary studies involving the use of NaCl/MEA plating media, injury was demonstrated through salt sensitivity. However the extent of this injury was considered insufficient for subsequent injury and repair studies. In the classical pattern of demonstration of microbial injury, monitored by paired plating media, differences in counts between paired plating media should ideally be at least 2-log cycles to be considered adequate. This was demonstrated (Table 1) using MEA plating media containing various concentrations of sugars (sucrose and glucose mixture) or glycerol, which showed that viable counts on glycerol/MEA at 0.87-0.94 a_w/pH 3.5 (lactic acid).

Additionally, major cause of injury to the low a_w (glycerol) stressed population might be possibly attributed to several factors such as dehydration, toxicity and osmostic stress. The dehydration caused by prolonged exposure to low aw (glycerol). Under the condition of dehydration, concentration of intracellular solutes will occur. This might have caused injury by speeding up rates of reaction not involving water, or cause large changes in internal pH as components of buffer systems are preferentially precipitated. This could subsequently cause denaturation of macro-molecules such as DNA, RNA and protein. On the other hand, the possible toxicity might caused by high glycerol concentration in the stress menstrum. Exposure to glycerol as an injury system comparable to freezing has also been reported earlier (Hunt and Busta, 1974; Faille *et al*., 2006). Moreover, osmotic stress encountered in the enumeration procedure when stressed cells from the low aw diluents identical to the stress system were plated onto high aw paired plating media can cause injury as well.

Conclusions

From the results of the present study, exposure of *Saccharomyces rouxii* YSa40 cells to the 0.64 a_w/pH 3.5 glycerol/CPB stress system was shown to cause injury as demonstrated by the extreme sensitivity of stressed cells to glycerol/lactic acid in the plating medium. The major cause of injury to the low a_w(glycerol) stressed population might be possibly attributed to several factors such as dehydration, toxicity and osmostic stress. Extrapolation of the results obtained in the present study to IMF implied that *Saccharomyces rouxii* YSa40 cells could also be injured in low pH fruit-based and glycerol-based IMF products. Such injury could possibly be compounded by mild heat treatment and by the addition of antimicrobial substances.

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