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Accelerated natural lactic fermentation of cereal-based formulas at reduced water activity

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Energy required to dehydrate fermented cereal-based food formulas plays a significant part in the production cost. Therefore the effect of reduced moisture content on the fermentation was investigated. Generally, lactic fermentation at reduced moisture content resulted in increased final pH. Significant acidification still occurred at 0.33 kg water/kg dry matter corresponding to either $a_w = 0.925$ in a sorghum-maize-soya (SMS) mixture, or $a_w = 0.950$ in a sorghum-maize-milk powder (SMM) mixture. Acidification adequate for microbiological safety ($\text{pH} \leq 4.5$) was achieved at 0.54 kg water/kg dm in SMS ($a_w = 0.950$) and 0.43 kg/kg dm in SMM ($a_w = 0.965$). In stable accelerated natural lactic fermentations obtained by inoculum recycling ('back-slopping') at 30°C, dominant lactic acid bacteria included *Lactobacillus plantarum*, *L. brevis*, *L. acidophilus* and *Lactococcus lactis*. Dominating yeasts were *Saccharomyces cerevisiae* and *Candida krusei*. *L. plantarum* isolated from high-moisture (1.5 kg/kg dry matter) SMS fermentations had higher specific growth rates than *L. plantarum* isolated from reduced moisture (0.54 kg/kg dm) SMS fermentations, when tested under similar a_w conditions. This effect was not caused by previous culturing conditions; probably different strains of *L. plantarum* having different a_w -optima dominate at high or reduced moisture conditions.

Key words: Sorghum; Maize; Soya; Skim milk powder; *Lactobacillus plantarum*; *Saccharomyces cerevisiae*; Water activity

Introduction

Solid substrate fermentation of food raw material is widely applied in the preparation of e.g. koji, tempe, sourdough and fermented maize foods including kenkey (Mensah et al., 1991) and mawè (Hounhouigan et al., 1992). Solid substrate fermentation was also applied successfully in the accelerated natural lactic fermentation of infant food formulas based on cereal-legume combinations (Nout et al., 1989a,b). Accelerated fermentation employs the principle of gradual enrichment of active acidifying strains of lactic acid bacteria through the recycling of inoculum ('back-slopping'). This is a simple technique which is particularly suitable for

household and small-scale commercial fermentation operations with only rudimentary facilities, taking place in, e.g., developing countries.

Previous experimental work with the accelerated fermentation technique was carried out using doughs consisting of equal weights of composite cereal-legume meal and water, resulting in 55–60% moisture content (total weight basis) or 1.2–1.6 kg water/kg dry matter (dm). Under these conditions the fermentation is highly effective and results in ≥ 2 pH units acidification within 6–8 h at 25–30°C. The microflora is dominated by lactic acid bacteria (*Lactobacillus plantarum*, *L. acidophilus*, *L. brevis* and *Lactococcus lactis*) with a consistent minority of yeasts (*Candida* and *Trichosporon* spp.) (Nout, 1991). For convenient storage and distribution, such fermented doughs must be dehydrated. The cost of energy required for dehydration contributes significantly to the overall cost of the product. On the basis of unpublished data, it can be estimated that at a fully occupied daily production capacity of 200 kg final dry product, the energy required to reduce the moisture content of fermented dough from 1.2 kg/kg dm to 0.05 kg/kg represents 20–25% of the total cost of ingredients, energy and equipment. However, with an initial moisture level of 0.5 kg/kg, drying energy represents 10–15% of the adjusted total cost. Consequently, important savings can be made if the fermentation stage is carried out at lower moisture contents.

The present investigation was carried out to test the influence of reduced moisture levels on the efficacy of accelerated natural lactic fermentation and on the growth of dominating microorganisms.

Materials and Methods

Ingredients

Ingredients included whole white sorghum (*Sorghum bicolor* ssp. *caffrorum*) and whole white 'mealy' maize (*Zea mays* (De Witte Molen, Meeuwen, The Netherlands), dry-dehulled yellow soya beans (*Glycine max*) (Kleinjan V.O.F., Rhoon, The Netherlands) and low-fat medium-heated spray-dried milk powder (CCF, Leeuwarden, The Netherlands).

Preparation of formulas

Sorghum and maize grains were cleaned dry and ground to whole-grain meals using a toothed disc mill (Condux type LV 15 M, Condux-Werk, Wolfgang bei Hanau, W-Germany). Soya beans were coarsely ground in the same mill. Formulas were designed to contain approx 13% (w/w) of metabolisable protein (Nout et al., 1989b). Formula SMS contained 370 g of sorghum meal, 370 g of maize meal and 260 g of soya bean meal per 1000 g. Formula SMM consisted per 1000 g of 400 g of sorghum meal, 400 g of maize meal and 200 g of milk powder.

Accelerated natural lactic fermentation of formulas

The principle of starting a stable accelerated natural lactic fermentation was described earlier (Nout et al., 1987, 1989b). For the first fermentation cycle, a

mixture of 100 g dry formula and the amount of tapwater required to achieve the desired moisture content, was incubated in a 450-ml screw-capped glass jar at 30°C for 24 h. Further fermentation cycles also lasted 24 h at 30°C and the mixtures consisted of 100 g dry formula, 10 g of the previously fermented batch and the weight of tapwater to achieve the desired moisture content.

Measurement of water relations

Resorption isotherm data were based on the dry matter content of samples (approx. 1 g) which had been equilibrated against saturated solutions of LiCl, MgCl₂, K₂CO₃, NaCl, KCl and BaCl₂ in vacuum desiccators at 30°C during 1 week. Sodium azide (0.1%) was used as a preservative. Dry matter contents were determined by vacuum oven drying at 50 or 70°C, and by Karl Fischer titration (Mettler DL18 Karl Fischer titrator). Equilibrium Relative Air Humidity (ERH) and water activity (a_w) of samples were measured using a Rotronic hygroscope (Rotronic AG, Zürich) and a Hygrometer Model a_w -box with measuring station EEJA (Novasina AG, Zürich), respectively.

Measurement of pH

Sampling and determinations were as described earlier (Nout et al., 1989a).

Microbiological analysis

Samples of 10 g were homogenized with 90 ml sterile peptone physiological salt solution (1 g peptone, 8.5 g NaCl, 1000 ml distilled water, pH 7.0 ± 0.2). Lactic acid bacteria were enumerated in pour-plates of de Man, Rogosa and Sharpe (MRS) medium (Merck No. 10661, Darmstadt, Germany) to which 12 g agar and 1 g natamycin ('Delvolid', Gist-Brocades, Delft, The Netherlands) were added per litre, with overlay, after incubation at 30°C for 3–5 days. The content of lactobacilli was estimated in pour-plates of Rogosa agar (Oxoid CM627) with overlay, after incubation at 30°C for 3 days. All lactic acid bacteria from MRS with natamycin were isolated and purified on MRS agar and maintained in MRS broth. Initial confirmation and grouping of lactic acid bacteria was based on Gram stain, catalase activity, growth at 15 and 45°C in MRS medium, gas from glucose and fermentation of lactose, sucrose, arabinose, sorbitol, cellobiose, fructose and mannitol added to glucose-free MRS medium. The square root of the number of colonies on the MRS agar with natamycin plate dilution chosen for enumerations were isolated and purified. Further characterisation of lactic acid bacteria was according to Sneath et al. (1986) based on the relevant biochemical reactions obtained with the 'API 50 CHL' system (API System SA, Montalieu Vercieu, France). Enumeration of yeasts and filamentous fungi was in pour-plates of Yeast Extract Glucose (YEG) Agar (Oxoid CM545) to which 0.01% w/v filter-sterilized oxytetracyclin (Pfizer) was added (OYEG). Incubation was at 25°C for 5 days. Isolations and purifications of yeasts were carried out on YEG agar as described above. Yeasts were grouped according to their morphology, and ability to sporulate on either V-8 agar (V-8 vegetable juice (Campbell Soup Co.) 100 ml, bakers' yeast 40 g, agar 2 g, distilled water 100 ml; pH 6.8; malt extract agar (MEA: malt

extract (Difco) 5 g, agar 3 g, dist. water 100 ml) or yeast malt extract agar (YMEA: malt extract 10 g, yeast extract 4 g, glucose 4 g, agar 15 g, distilled water to 1000 ml, pH $\pm$ 7.3), their ability to ferment glucose, grow at 37°C and utilise KNO₃ or lysine as sole source of nitrogen (Campbell and Duffus, 1988). Further characterisation was carried out using the 'ID 32 C' system (API System SA, Montalieu Vercieu, France).

Enterobacteriaceae were enumerated in pour-plates of Violet Red Bile Glucose (VRBG, Oxoid CM 485) medium with overlay after incubation at 37°C for 24 h.

The water activity (a_w) of diluent and microbiological media was adjusted to that of the sample by adding amounts of glycerol or sorbitol calculated according to the Norrish, equation (Norrish, 1966).

Growth kinetics

Growth curves of *L. plantarum* were measured in a conical flask containing 100 ml MRS broth, after inoculation with 1 ml of a 24-h old MRS culture, and incubated in a water bath held at 30°C. Growth curves of *Saccharomyces cerevisiae* were measured in 100 ml yeast extract glucose broth (YEGB: yeast extract 5 g/l, glucose 20 g/l) at 30°C after inoculation with 0.1 ml of a 10⁻³ dilution of a 24-h YEGB culture. Growth was monitored as extinction values at 645 nm in a spectrophotometer (Beckman DU-64), or as viable count in MRS agar or YEG agar, respectively.

All experiments were carried out in duplicate; all analyses in triplicate. Mean values are presented.

Results

As the water activities caused by addition of water prior to fermentation are of relevance in this study, only the resorption data of the water sorption isotherms of the formulas are given. Table I shows the a_w resulting from resorption of SMS and SMM formulas at 30°C.

The effect of the moisture content on the extent of acidification is shown in Fig. 1 (SMS) and Fig. 2 (SMM). No acidification took place in SMS at 0.25 kg moisture/kg dm, but significant acidification occurred at 0.33 kg/kg dm and higher moisture contents. At all tested moisture levels, it took 4–6 daily inoculum recyclings at 10% inoculation rate, to obtain a stable extent of acidification. The a_w had a significant effect on the final pH. Table II shows that in all treatments in which acidification took place, the number of lactic acid bacteria was not significantly different. Lactobacilli were the dominant group of lactic acid bacteria. *Lactobacillus plantarum* dominated at all tested moisture levels. *L. brevis* was found particularly at 0.33 kg water/kg dm. *L. acidophilus* and *Lactococcus lactis* were present as a minority in most samples. Also, the number of yeasts was not affected by the moisture content in the range 0.33–0.82 kg/kg dm. *Saccharomyces cerevisiae* was dominating and found in all samples, whereas *Candida krusei* occurred in SMM at reduced moisture levels. The Enterobacteriaceae were de-

TABLE I

Water activity at 30°C of SMS^a and SMM^b formulas at different moisture contents

Moisture content (kg water/kg dry matter)	Water activity (a_w)	
	SMS	SMM
0.1365	0.755	
0.1678	0.845	
0.2500	0.885	
0.3330	0.925	0.960
0.4286	0.940	0.965
0.5385	0.970	0.975
0.6667	0.975	0.980
0.8182	0.990	0.990
1.0000	0.990	
1.5000	0.990	

^a SMS, sorghum-maize-soya formula (370, 370 and 260 g respectively, per kg).^b SMM, sorghum-maize-skim milk powder formula (400, 400 and 200 g, respectively, per kg).

tectable only in fermented formulas with 0.25 and 0.33 kg moisture/kg dm. At higher moisture levels, the lower pH values apparently caused their death.

From stabilized fermentations of SMS with 1.5 kg water/kg dm and 0.54 kg water/kg dm, several isolates of *L. plantarum* were obtained and compared on the

Fig. 1. Effect of moisture content on acidification in SMS formula. ◇—◇, Moisture content (kg water/kg dry matter) 0.25, a_w 0.885; △—△, 0.33, 0.925; ○—○, 0.43, 0.940; □—□, 0.54, 0.970; ▲—▲, 0.82, 0.990.

Fig. 2. Effect of moisture content on acidification in SMM formula. Δ — Δ , Moisture content (kg water/kg dry matter) 0.33, a_w 0.955; $\circ$ - - - $\circ$, 0.43, 0.965; $\square$ - - $\square$, 0.54, 0.975; $\blacktriangle$ — $\blacktriangle$, 0.82, 0.990.

basis of their profile of assimilated carbohydrates in the API50CHL test. Of these, 3 isolates with identical biochemical profiles were selected. Strain 4 had been isolated from SMS with 1.5 kg water/kg dm using MRS agar. Strains 1G and 1S had been isolated from SMS with 0.54 kg water/kg dm using dilution liquid and MRS agar to which respectively glycerol (270 g glycerol to 1000 g MRS agar) and sorbitol (520 g sorbitol to 1000 g MRS agar) had been added to achieve a_w 0.947, i.e. comparable to the low-moisture SMS from which they originated. None of these strains were able to assimilate glycerol or sorbitol as a C-source, when added to sugar-free MRS broth. Fig. 3 shows the growth curves of these strains on MRS broth, MRS + sorbitol (MRSS; a_w 0.947) and MRS + glycerol (MRSG; a_w 0.947). Although quite similar optical densities were finally reached in the stationary phase, strain 4 always had a higher growth rate in the logarithmic phase. In addition, glycerol had a stronger inhibitory effect than sorbitol at a_w 0.947. In order to assess whether this effect was due to the different previous cultivation history of these cultures, strain 4 was grown under different conditions by sub-culturing thrice in MRS, MRSS and MRSG. Subsequently, the pre-cultured cells were inoculated into MRS, MRSS and MRSG. No significant influence of previous history of strain 4, on its growth rate in MRS, MRSS or MRSG were observed (data not included). The inhibitory effect of glycerol was again stronger than that of sorbitol.

Similarly, isolates of *Saccharomyces cerevisiae* were obtained from SMS with 1.5 kg water/kg dm (strain 'O' isolated on OYEG agar) and SMS with 0.54 kg

Fig. 3. Growth of *Lactobacillus plantarum* isolates of different origin. $\diamond$ — $\diamond$, *Lactobacillus plantarum* strain 4, growth medium MRS; $\triangle$ — $\triangle$, strain 4, growth medium MRSS (MRS + sorbitol added to $a_w = 0.945$); $\circ$ — $\circ$, strain 4, growth medium MRSG (MRS + glycerol added to $a_w = 0.945$); $\blacklozenge$ — $\blacklozenge$, strain 1G, growth medium MRS; $\blacktriangle$ — $\blacktriangle$, strain 1G, growth medium MRSS; $\bullet$ — $\bullet$, strain 1G, growth medium MRSG; ∇ — ∇ , strain 1S, growth medium MRS; $\diamond$ — $\diamond$, strain 1S, growth medium MRSS; $\square$ — $\square$, strain 1S, growth medium MRSG.

water/kg dm (strains 'O-G' and 'O-S' isolated on OYEG agar with addition of glycerol or sorbitol, respectively, to give a_w 0.945). The growth of the isolates mentioned having identical biochemical profiles with the ID32C test was monitored on YEG broth (YEGB; a_w ca. 1.0), YEG broth + glycerol (YEGBG; a_w 0.947) and YEG broth + sorbitol (YEGBS; a_w 0.947). The growth rate of the respective isolates on each of the media did not differ significantly (results not shown). However, in contrast to the *L. plantarum* isolates, *Sacch. cerevisiae* was more strongly inhibited by sorbitol than by glycerol at the same a_w (results not shown).

Discussion

The resorption data shown in Table I are of the order of magnitude of those reported of cereals and pulses (Krischer and Kast, 1978; Jowitt et al., 1983). However, most published sorption isotherms are less reliable at $a_w > 0.97$ (Jowitt et al., 1983). SMS was less hygroscopic compared with SMM. Consequently, if skim milk powder is available at reasonable cost, it will have the economic advantage that the fermentation can take place at lower moisture levels than in SMM formula.

TABLE II

Microbiological content of SMS^a and SMM^a formulas before fermentation and after stabilized accelerated natural lactic fermentation at different moisture contents

	Lactic acid bacteria	Lactobacilli	Yeasts	Micro-bacteriaceae
SMS formula:				
Unfermented, dry	2.8 ^b	2.3	3.2	2.4
Fermented ^c at:				
0.25 kg water/kg dm	3.11	2.30	5.65	2.73
0.33 kg water/kg dm	9.32	9.33	7.25	3.97
0.43 kg water/kg dm	9.78	9.77	7.62	< 1.7
0.54 kg water/kg dm	9.48	9.65	7.14	< 1.7
0.82 kg water/kg dm	9.85	9.87	7.44	< 1.7
SMM formula:				
Unfermented, dry	2.3	2.0	3.1	3.6
Fermented ^c at:				
0.33 kg water/kg dm	9.34	9.36	6.47	2.19
0.43 kg water/kg dm	9.60	9.61	6.92	< 1.7
0.54 kg water/kg dm	9.39	9.61	6.85	< 1.7
0.82 kg water/kg dm	9.48	9.72	6.95	< 1.7

^a For explanation see Table I.

^b Log₁₀ N g⁻¹.

^c 7 daily recyclings at 10% inoculation rate, incubated at 30°C.

A major reason for fermenting mixtures of cereals and legumes is to improve the microbiological safety of weaning foods. Dehydration of the fermented product is required for convenient packaging, shelf-life and distribution. In view of the relatively high cost of thermal energy required for dehydration, the fermentation should be carried out at a minimum effective moisture level. A pH ≤ 4.5 is regarded as essential to ensure the inhibition or death of undesired bacteria and to prevent the germination of spores from pathogenic Bacillaceae. From the results in Figs. 1 and 2 it may be concluded that in the tested mixtures, the above requirement is achieved at 0.43 kg water/kg dm in SMM, and at 0.54 kg/kg dm in SMS. Compared with fermentations at a relatively high moisture level (1.5 kg/kg cm), the reduction of the moisture content did not significantly affect the levels of functional microflora (mainly lactic acid bacteria).

At all tested moisture levels, *L. plantarum* was the dominating species of lactic acid bacteria. The minimum a_w enabling growth of microorganisms depends on many factors, including the nature of the a_w controlling solute (Brown, 1976) and the pH (Farkas et al., 1988). Suppressed with NaCl, the minimum a_w for growth of *L. plantarum* is 0.94–0.97 (Imhoff, 1986; Farkas et al., 1988) but with glycerol it is 0.885–0.915 (Rechacek et al., 1982; Imhoff, 1986). Fig. 1 and Table II show that at a_w 0.883 (in SMS formula) no appreciable growth or acidification occurred.

Although our *L. plantarum* strains did not assimilate sorbitol, the species is listed as sorbitol-positive (Sneath et al., 1986) and consequently sorbitol must enter

the cell. However, the transport mechanism of sorbitol into cells of lactobacilli has not been reported. The consistent difference in growth rate between sorbitol- and glycerol-depressed a_w in MRS (Fig. 3) must be caused at intracellular level. Either glycerol has a specific inhibitory effect, or sorbitol acts as an osmoprotectant or compatible solute. At a_w near to 1.0 (MRS) and a_w 0.945 (MRSG; MRSS), *L. plantarum* strain 4 isolated from SMS with 1.5 kg moisture/kg dm had a higher growth rate than *L. plantarum* strains 1G and 1S isolated from SMS with 0.54 kg moisture/kg dm. This effect was not caused by previous culturing conditions. Consequently, we suggest that biochemically identical strains of *L. plantarum* may still be different on the basis of their growth rate and that high moisture levels enable the enrichment of such relatively fast-growing strains.

Lowering the moisture content in the formulas did not affect yeast growth except at a_w 0.883 in SMS. In any case, *Sacch. cerevisiae* is able to grow at lower a_w than *L. plantarum*. With a_w adjusted with glucose $a_w \geq 0.89$ was required for growth (Cerrutti et al., 1990), but with glycerol $a_w \geq 0.80$ was reported (Rehacek et al., 1982).

A possible risk of extreme reduction of moisture levels in fermenting formulas is that yeasts will outnumber lactobacilli. Table II shows that this happens indeed at 0.25 kg water/kg dm in SMS. However, this moisture level is of less relevance since it does not achieve the aim of acidification. At all higher moisture contents tested, yeasts represented less than 1% of the number of lactobacilli.

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