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Consistency, Polysaccharidase Activities and Non-Starch Polysaccharides Content of Soya Beans During Tempe Fermentation

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Abstract: The relation between consistency of soya beans, polysaccharidase activities and the non-starch polysaccharides (NSP) content of soya beans was investigated during tempe fermentation. The fermentations were carried out in a rotating drum reactor (RDR) as well as in the traditional stationary tempe process. The firmness of the soya beans decreased rapidly during the first day of incubation. At increased incubation temperatures the hardness of the beans decreased more rapidly. In the RDR glycosidase activities became significantly higher beyond 48 h of incubation compared with the traditional process. This might have resulted from (a) the better control of temperature and gas composition in the RDR, and/or (b) the agitation in the RDR. The content of arabinose, galactose and uronic acids in the water-unextractable solids of NSP decreased more rapidly than that of glucose, mannose, xylose and fucose. These results indicate that during enzymatic maceration of soya beans in tempe fermentation, the arabinogalactan and pectin fractions are preferentially solubilised.

Key words: tempe, consistency, *Rhizopus oligosporus*, polysaccharidases, non-starch polysaccharides, solid–substrate fermentation.

INTRODUCTION

Tempe is a traditional Indonesian fermented food which consists of cooked, dehulled soya beans, or other materials, bound into a solid cake by the growth of a mould, most commonly *Rhizopus oligosporus* (Nout and Rombouts 1990). In good-quality tempe the bean cotyledons are bound together sufficiently tightly to allow the product to be diced or sliced before frying.

Blakeman *et al* (1988) compared three methods for assessing fungal biomass in tempe. They found that the force required to draw a pin through tempe gave a good indication of the degree of mycelial growth and substrate binding and suggested that this was a more useful measure than direct determinations of fungal biomass

after enzymic digestion of the substrate or than assaying for *N*-acetyl glucosamine.

Nout *et al* (1985) stated that uncooked tempe should have a consistency corresponding to a force of 1.5–2.5 N required to break a cylinder of tempe 50 mm long and 15 mm in diameter suspended at its extremities. This procedure is only applicable to well-developed tempe and cannot be used to distinguish hyphal growth from the changes in consistency of the soya beans.

Ariffin *et al* (1994) described a quantitative method for measuring the strength of the hyphae binding the cotyledons together in soya bean tempe. The force to failure and the work to failure both increased from zero values before hyphal development to maximum values after about 30 h of incubation and then declined as the tempe aged and the hyphae started to deteriorate.

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Unfortunately, the authors neglected, without experimental evidence, the influence of the consistency on their results.

Sarrette *et al* (1992) described the production and activity of polysaccharidases, notably polygalacturonase, cellulase and xylanase, by *Rhizopus oligosporus* during tempe fermentation. The maximum activity for all polysaccharidases tested occurred 20–30 h after inoculation.

The edible dry matter portion of soya beans consists of protein (380 g kg⁻¹), total lipids (240 g kg⁻¹), carbohydrates (310 g kg⁻¹), fibre (30 g kg⁻¹) and ash (40 g kg⁻¹) (MacCarty and Matthews 1984). The carbohydrate components can be divided into monosaccharides (minor quantities), oligosaccharides (100–150 g kg⁻¹ dry matter) (DM) and polysaccharides (130–220 g kg⁻¹ DM) (Fennema 1976). Important components which are related to the consistency of the soya beans are the plant cell wall polysaccharides. The cell wall polysaccharides can be divided into water-extractable polysaccharides and water-unextractable polysaccharides.

Several studies have focused on the isolation and characterisation of cell wall polysaccharides from soya beans. Arabinose, galactose, glucose and galacturonic acid are the prevalent sugar residues, in addition to smaller amounts of fucose, rhamnose, xylose and mannose (Brillout *et al* 1988; Schols *et al* 1993).

In the present study we investigated the production and activities of polysaccharidases in relation to the consistency of tempe. The fermentations were carried out with *Rhizopus oligosporus* at 25, 30 and 37°C in the traditional process. The results were compared with those obtained in a rotating drum reactor (RDR) which was described previously by De Reu *et al* (1993). In this laboratory-scale bioreactor, temperature control during solid-substrate fermentation can be achieved with discontinuous or continuous rotation and forced evaporation by aeration. As a result of the rotation a granular product is obtained, in contrast with the cake-like solid mass resulting from the traditional process.

MATERIALS AND METHODS

Organism

Rhizopus microsporus var *oligosporus* strain LU 575 (NRRL 5905) was grown and maintained at 30°C on Malt Extract Agar (Oxoid, CM 59, UK). Sporangiospore suspensions were obtained by scraping off the sporangia from a culture after 7 days incubation at 30°C, and suspending them in sterile distilled water containing Tween 80 (1 ml litre⁻¹) (Merck, Germany). The viable count varied between 5×10^5 and 10^6 cfu ml⁻¹, when determined on Rose-Bengal Chloramphenicol

Agar (Oxoid CM549). The sporangiospore suspensions were used immediately after preparation.

Tempe manufacturing process

Dehulled yellow-seeded soya beans (*Glycine max* L) were soaked overnight using the accelerated acidification method (Nout *et al* 1987). Subsequently, the beans (pH soak water < 4.2) were washed with tap water and boiled for 20 min, cooled, superficially dried (15–30 min, at room temperature) and inoculated with sporangiospore suspension (10 ml kg⁻¹).

Petri dishes (ϕ 9 cm; 6 side perforations, ϕ 1 mm) were filled with 60 g inoculated beans, sealed with tape and incubated at 25, 30 or 37°C. An autoclaved RDR (De Reu *et al* 1993) was filled with 1 kg of cooked and inoculated soya beans. The reactor was placed in a temperature-controlled incubator at 30°C. During the experiments a discontinuous rotating scheme was used to avoid substrate temperatures rising beyond 36°C. After the substrate temperature reached 36°C, the reactor vessel was rotated alternately clockwise (6 s at 8 rpm) and counter-clockwise (6 s at 8 rpm) for 1 min. Due to the rotation, the temperature of the substrate dropped by 1.5–2.0°C. After rotation the reactor vessel was kept stationary until the substrate temperature reached 36°C again. Then the rotation cycle was repeated. This procedure resulted in a saw-tooth temperature pattern (De Reu *et al* 1993).

Samples for biochemical analysis were taken at various time intervals and immediately frozen in liquid nitrogen. The samples were stored at –20°C, lyophilised and stored at –20°C in the dark until analysis. Although incubation is stopped after 30–48 h under production conditions, we continued the fermentation in the static samples for up to 144 h to observe the effects of on-going biochemical processes. After 72 h of incubation the material inside the RDR was removed and stored at 37°C.

Consistency

To determine the consistency of the soya beans, 10–15 cotyledons were dissected carefully from the fermenting cake and placed in a Mulder device (Mulder 1946). With this system the force required to decrease the height of the individual cotyledons by 50% was determined. The Student's *t*-test (Owen 1962) was used to determine whether differences were significant.

Enzyme extraction

The tempe samples were ground and frozen in liquid nitrogen, and stored at –80°C until analysis. Enzymes were extracted by adding 9.5 or 9.0 ml water to 0.5 or

1.0 g of tempe, respectively, depending on the determined enzyme level of a previous trial.

Enzyme assays

Activities of enzymes were assayed by determination of reducing sugars released by the hydrolysis of polygalacturonic acid (Cat no 102711, ICN Pharmaceuticals, USA) for polygalacturonase activity; linear araban (NOVO, Denmark) for arabinase activity; birch xylan (Roth GmbH & Co, Germany) for xylanase activity and carboxymethylcellulose (Akucell AF0305, AKZO, The Netherlands) for endocellulase activity. A mixture of 250 μl , containing 50 μl substrate (5 g litre⁻¹ in water), 25 μl test sample and 175 μl 50 mM sodium acetate buffer, pH 5.0, was incubated for 1 h at 30°C. The release of reducing sugars was determined by the Nelson Somogyi method (Spiro 1966) using a Beckman DU-64 spectrophotometer at 520 nm (Beckman Instruments, Fullerton, CA, USA).

Activities of α -D-xylopyranosidase, β -D-xylopyranosidase, α -D-glucopyranosidase, β -D-glucopyranosidase, α -D-galactopyranosidase, β -D-galactopyranosidase, α -D-mannopyranosidase (Koch-Light, Colnbrook, Bucks, UK; Cat nos 14 308, 14 303, 14 279, 14 280, 14 301, 14 277 and 14 285, respectively), α -L-arabinopyranosidase and α -L-arabinofuranosidase (Sigma, USA, Cat nos N3512 and N3641, respectively) were measured using their corresponding *p*-nitrophenyl (PNP) derivatives as substrates. An aliquot of 100 μl test sample was added to 25 μl PNP-substrate (1 g litre⁻¹ in 50 mM sodium acetate buffer, pH 5.0) and incubated for 1 h at 30°C. The reaction was stopped by adding 125 μl of 50 mM glycine buffer, pH 9.0 with 2 mM EDTA. The released PNP was measured at 405 nm, and quantified using a molar absorption coefficient of 13 700.

All activities were expressed in international units (U; $\mu\text{mol min}^{-1}$). Measurements of enzyme activities were carried out in duplicate.

Non-starch polysaccharides

The total non-starch polysaccharides (NSP) content of the water-unextractable solids (WUS) fraction was estimated by adding the uronic acids content and the neutral sugar content.

Uronic acid content

The anhydro-uronic acid content of the WUS fraction was determined by the automated *meta*-hydroxydiphenyl assay (Thibault 1979).

Neutral sugars

Neutral sugars in the WUS fraction of tempe were analysed as non-starch polysaccharides (Englyst and Cummings 1984) using inositol as internal standard and pretreatment with H₂SO₄ (720 g kg⁻¹) for 1 h at 30°C prior to hydrolysis with 1 M H₂SO₄ for 3 h at 100°C.

After starch hydrolysis, extraction and washing of the lyophilised tempe, according to Englyst and Cummings (1984) and Englyst *et al* (1992), WUS was obtained. The WUS fraction was hydrolysed. Alditol acetates were obtained after reduction and acetylation according to the method described by Englyst and Cummings (1984). Alditol acetates were separated using a DB1701 column (30 m, 0.32 mm ϕ) in a Carlo Erba GC (Carlo Erba Strumentazione, Rodano, Milan, Italy; type HRGC 5160) operated equipped with a flame ionisation detector.

Chromatography conditions were as follows: sample size, 1 μl ; carrier gas, helium; pressure, 100 kPa; the temperature was programmed from 80 to 180°C at a rate of 2°C min⁻¹ and from 180 to 270°C at a rate of 1.5°C min⁻¹ and was kept for 3 min at 270°C.

RESULTS

Consistency

In Fig 1 the effects of incubation temperatures and the incubation period on the consistency of soya beans are shown. After soaking, a force of 28.9 ± 5.0 N was required to reduce the height of the cotyledons by 45–50%; subsequent cooking reduced the required force to 25.5 ± 4.9 N. During the first day of incubation the required force decreased rapidly from 25.5 ± 4.9 N to 13.6 ± 4.6 N, 11.0 ± 4.6 N, 10.1 ± 3.2 N and 7.3 ± 1.4 N at 25, 30, 37°C and in the RDR (36°C), respectively. During further incubation this order was maintained. The decrease in the required forces was less marked after the first day.

The firmness of the RDR-fermented material was significantly less ($P < 0.05$) than that of the traditionally fermented product, except after 48 h of incubation. Between 25 and 30°C, no significant differences ($P > 0.05$) were observed after 1, 2 and 3 days of incubation. The consistency of the beans fermented at 30°C and 37°C became significantly different ($P < 0.05$) beyond 72 h of incubation.

Enzyme activity

In Table 1 the enzyme activities during the fermentation of soya beans with *R. oligosporus* are shown for the traditional fermentation at 25, 30 or 37°C and for the agitated process in the RDR. The total enzyme activities (TEA) in the RDR and at 37°C were similar after 48 h

Fig 1. The consistency of tempe as a function of the incubation time. Fermentations took place in the traditional system at 25, 30 and 37°C and in the rotating drum reactor at 36°C. The results on the y-axis represent means and standard deviation ($n = 10-15$). $T = -4$ h represents soaked beans. $T = 0$ h represents cooked soya beans after inoculation.

of incubation. During the third day of incubation the TEA increased in the RDR, whereas the TEA at 37°C decreased.

If the TEA is divided into enzyme activity on polymers and that on glycosides some interesting differences are observed. In the traditional non-agitated fermenta-

tions the ratio depolymerases : glycosidases increased rapidly up to 72 h of incubation. In other words, in this system activity on polysaccharides was more important than glycosidase activity. If the incubation temperatures were compared, a significant increase in TEA was observed at 37°C compared with 25 or 30°C. When the

TABLE 1

Enzyme activities (U g^{-1} lyophilised tempe) during the fermentation of soya beans in the stationary traditional process at 25, 30 and 37°C and in an agitated rotating drum reactor (RDR) at 36°C

	RDR (36°C)				25°C			30°C			37°C		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
<i>Activity on polymers (I):</i>													
Arabinase	1	-1 ^a	5	0	1	2	6	0	1	2	-2 ^a	10	10
Cellulase	1	0	5	2	0	2	1	1	1	3	3	8	3
Polygalacturonase	1	6	5	5	2	2	3	1	1	3	7	5	8
Xylanase	1	3	4	3	1	1	1	1	1	3	5	7	6
Subtotal	4	9	19	10	4	7	11	3	4	11	13	30	27
<i>Glycosidase activities (II):</i>													
α -Galactosidase	1	4	5	11	3	2	2	2	4	2	5	4	3
β -Galactosidase	1	4	4	7	3	2	2	1	3	2	4	3	2
α -Xylosidase	1	3	4	6	4	2	4	3	4	5	3	3	3
β -Xylosidase	1	4	4	7	3	2	2	2	4	2	3	3	2
α -Mannosidase	1	2	4	7	3	3	2	1	3	2	3	3	2
α -Glucosidase	1	2	4	7	3	2	2	2	4	2	4	3	2
β -Glucosidase	1	3	4	7	3	2	2	2	4	2	4	3	2
Arabinofuranosidase	1	3	4	10	3	2	1	1	3	2	3	2	2
Arabinopyranosidase	1	3	3	7	3	2	2	1	4	2	3	2	2
Subtotal	9	28	36	69	28	19	19	15	33	21	32	26	20
Total	13	37	55	79	32	26	30	18	37	32	45	56	47
Ratio (I)/(II)	0.21	0.29	0.51	0.16	0.12	0.31	0.73	0.19	0.17	0.59	0.44	1.17	1.47

^a Negative values were obtained due to correction for blank results.

individual depolymerases or glycosidases were compared, only small differences could be detected.

Non-starch polysaccharides

In Table 2 the neutral sugars and uronic acid contents of soya beans are shown for the fermentation with *R oligosporus* at 25, 30 or 37°C in the traditional stationary system, as well as in the agitated RDR. During incubation a drop in total NSP was observed. The higher the incubation temperature the lower the level of

NSP in the WUS fraction at the end of the incubation. The differences in NSP content between the RDR and the traditional system at 37°C were very small.

Consistency vs enzyme activities and NSP content

In Fig 2 the relation between total polysaccharidase activities and the force (N) required to give a reduction of 50% in height of the cotyledons is shown. The higher the enzyme activity the less was the force required for this reduction. Compared to the firmness of cooked inoculated beans a significant decrease of the force

TABLE 2
Non-starch polysaccharides content ($\mu\text{mol g}^{-1}$ water-unextractable solids) during the fermentation of soya beans in the stationary traditional process at 25, 30 and 37°C and in an agitated rotating drum reactor (RDR) at 36°C

	RDR (36°C)				25°C			30°C			37°C		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Arabinose	339	263	187	194	383	243	263	284	262	220	319	195	163
Fucose	48	44	35	36	54	44	50	48	46	43	47	38	34
Galactose	625	497	356	350	684	477	518	548	496	429	595	387	350
Glucose	420	563	430	405	648	554	613	486	658	546	574	386	358
Mannose	43	45	43	43	49	44	45	46	45	42	44	35	33
Rhamnose	32	26	18	19	36	24	27	30	25	22	29	20	16
Xylose	139	134	94	96	171	123	129	127	140	114	145	91	81
Uronic acids	72	16	16	10	57	54	57	69	52	54	46	39	27
Total:	1718	1588	1179	1053	2082	1563	1702	1638	1724	1470	1799	1191	1062

Fig 2. Relationship between total polysaccharidase activities (U g^{-1}) and the force (N) required to give a reduction of 50% in height of the soya cotyledons. Results represent pooled data of experiments carried out in the RDR and the traditional fermentation system. (□) Fermented samples.

required resulted from very low enzyme activities. A maximum r^2 value of 0.77 was obtained when the data were fitted to an exponential curve.

In Fig 3 the relation between the NSP-content ($\mu\text{mol g}^{-1}$ WUS) of the soya beans and the force (N) required to obtain a 50% reduction in height is shown. Increases above the initial value of $1718 \mu\text{mol NSP g}^{-1}$ WUS might be due to shifts in the composition of the WUS fraction, eg protein becomes more soluble during fermentation (De Reu 1995). The lower the NSP content, the less was the force required, indicating a less firm structure.

DISCUSSION

In tempe research little attention has been paid to polysaccharidases and their effects on the consistency of soya beans. It is expected that during fermentation the hyphae may soften the soya beans by enzymatic degradation of cell walls and mechanically pushing the cells apart. This expectation is supported by histological observations of growth of *R oligosporus* on soya beans by Jurus and Sundberg (1976). Penetration and growth of the hyphae were generally inwardly directed and perpendicular to the surface of the cells. The degree of distortion caused by the fungus was most severe at or near the cotyledon surface. Portions of the outermost cell

layers of the cotyledon were often completely permeated with mycelium, creating an indistinct mass. The walls of these cells were shrivelled, the cytoplasm was very distorted, and frequently tissue was so grossly disrupted that individual cells were no longer recognisable. Fewer hyphae and little or no distortion were observed among the inner cellular layers of the cotyledon (Jurus and Sundberg 1976). After penetration of the outer layers of the cotyledons, the energy sources in the inner tissue, eg lipids (De Reu *et al* 1994) and proteins become accessible to hydrolysis by lipases and proteases, respectively.

The major reduction in consistency was observed during the first day of incubation, which implies that during the initial growth stages of fungal development mechanical and enzymatic maceration are very effective. It seems that cell wall polysaccharides which are involved in maintaining the structural integrity of the beans could be hydrolysed easily during the first 24 h of incubation. After 24 h some specific parts of the remaining structure were solubilised, without decreasing the consistency a lot. Those specific parts consist of arabinose, galactose and uronic acids as shown in Table 2. This indicates that the arabinogalactan and pectin fractions were preferentially solubilised.

As described earlier (De Reu *et al* 1993), the RDR enables solid-substrate fermentations to be conducted under controlled conditions of temperature and oxygen supply by avoiding heat and mass transfer limitation problems. As expected, growth-limiting conditions were

Fig 3. Relationship between non-starch polysaccharides content of soya beans ($\mu\text{mol g}^{-1}$ water-unextractable solids) and the force (N) required to give a reduction of 50% in height of the soya cotyledons. Results represent pooled data of the experiments carried out in the RDR and the traditional fermentation system. ($\diamond$) Cooked inoculated beans, and ($\square$) fermented samples.

rapidly reached in the traditional packed-bed systems. After 48 h of incubation the total enzyme activity increased rapidly in the RDR while in the traditional systems maximum enzyme activity had already been passed.

In the RDR consistency changed as a function of both enzyme activity and shear forces during agitation, and therefore it is not surprising that the hardness was less than in the product of stationary fermentation. However, we were not able to distinguish whether this was due to the higher total enzyme activity or to the effect of shear forces or a combination of both.

The reduction of arabinose, galactose and uronic acids (Table 2) shows that during enzymatic maceration arabinogalactan and pectin were the predominant fractions solubilised. During tempe fermentation, growth of *R. oligosporus* on soya beans resulted in complex biochemical and physical changes in the beans. The results might be used to form a link between previous histological observations and cell wall structure studies. The observed changes improve the fundamental knowledge about the tempe process and could be used to optimise the production process, in particular in determining the required incubation time.

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