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Influence of most-probable-number method and container perforation numbers on fungal population density during tempeh production

SUBRATA NATH BHOWMIK[1], S BALASUBRAMANIAN[2] and D N YADAV[3]

Central Institute of Post-Harvest Engineering and Technology, Ludhiana 141 004

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Tempeh is a collective name for a sliceable mass of precooked fungal fermented beans or some other food processing by-products bound together by the mycelium of a living fungus (mostly *Rhizopus* spp.) (Nout and Kiers 2005). Tempeh has gained world-wide acceptability for its multiple health benefits. Generally, a good quality fresh tempeh is a compact and sliceable mass of processed raw material covered, penetrated and held together by dense nonsporulated mycelium of the fungus.

Several containers made of leaf, plastic, wood or stainless steel is used eventually for tempeh production at cottage and industrial level. The limited air in a closed tempeh container significantly altered the tempeh quality at harvest (Aoki *et al*. 2003, Steinkraus 1996). Perforation of container for aeration upon maintaining adequate moisture in the raw substrate is a technological intervention to expedite tempeh production (Wadud *et al*. 1986). Hence perforation number as function of container size may be critical for fungal proliferation in scaling up of tempeh commercially. However reports on effect of container perforation on tempeh production are meagre.

In our preliminary study, the effect of different number of container perforations on the growth of fungus during tempeh production under variable incubation period is reported. The objective is to determine the optimum fungal growth needed for tempeh formation. The most-probable-number (MPN) method much restricted to bacterial viable cell counting presently finds scope for fungal enumeration as well. This technique allows accurate counting because the broth can be incubated for a longer time (Griffith *et al*. 2009, McGranaghan *et al*. 1999, Obispo and Dehority 1992). Hence MPN technique was used for the first time to evaluate its suitability in fungal food research. Additionally textural attributes of tempeh were recorded to assess its maturity (Ariffin *et al*. 1994).

Acidified potato-dextrose (pH 3.5) medium (HiMedia Laboratories Pvt Ltd, India) selective for fungal isolation was used in this investigation.

The fungus, viz. *Rhizopus oryzae* (ITCC 7382.09) was grown on 10 ml acidified potato-dextrose-agar in 100 ml Erlenmeyer flasks at 30±2°C for 2 days. The spore suspension was prepared by washing the growth surface for three times with approximately 30 ml of sterile 0.9% (w/v) NaCl and was stored at 4ºC. The spore concentration was assessed with haemocytometer.

Twenty disposable plastic petridishes of equal dimension (88 mm × 13 mm) were selected as container for tempeh fermentation. Different number of perforation (3, 4, 5) of 0.5 mm diameter at bottom and 4 perforations along the holding plate side of petridish at a distance of 4 cm and 6.9 cm were made, respectively. Thus, the petridish treatments with 7, 8 and 9 perforations were maintained. A set of petridishes without any perforation was maintained as control.

Soybean tempeh was produced with modifications (Nout *et al*. 1987). The whole soybean (600 g) from local market was soaked in tap water (1: 3) for 16 hr at room temperature. After dehulling, the soybean was boiled for 30 min in excess water and cooled. Thirty gram of soybean was packed into the petridishes were inoculated with *R. oryzae* (@10^4 spores/g) in laminar air-flow hood (Feng *et al*. 2005). A set of five petridishes was stacked one above the other maintaining a gap of 2 mm for free air passage in dark at 35±2ºC.

The fungal population density of soybean tempeh was examined at 0, 12, 24, 36 and 48 hr of incubation period by most-probable-number (MPN) method upto 1:10^6 dilution in 0.9% (w/v) NaCl water. From each dilution of samples, 1ml aliquot was inoculated in 5 dilution replicate tubes (Fisherbrand Disposable Culture Tubes Borosilicate Glass, 12 mm × 75 mm size) containing 2 ml acidified potato-dextrose broth. The positive tubes with mycelial growth

[1] Senior Scientist (e mail: snb70@yahoo.co.in), [2] Senior Scientist, [3] Senior Scientist, FG&OP Division

were noted at 72 h (30±2°C) incubation and population density was expressed as colony-forming units (CFU)/g on dry weight basis (Cochran 1950). For validation of MPN results, spread plate (SP) assay were conducted concomitantly in petridishes (90 mm) containing solidified acidified potato-dextrose agar (@15 ml/petridish). The pH and visual observations of tempeh were recorded for each treatment.

Texture profile analysis (TPA) test was performed for tempeh using texture analyser (Model TA-HD*i*, Stable Micro Systems Ltd, Vienna Court, Lammas Road, Godalming, Surrey GU71YL, UK). A crosshead speed of 1 mm s^{-1} with a 5 kg compression load cell of 75 mm compression probe was performed for compressing tempeh sample. A slab of tempeh 15 × 15 × 13 mm was placed through the descended frame and across the underlying slot. The sample was subjected to 30% strain with a time gap of 2 s between the compressions. The degree of compression was selected based on the preliminary experiments. For young tempehs (< 24 hr age), not having sufficient hyphal development to hold the beans were not subjected to texture analysis. Standard TPA parameters, viz. firmness, cohesiveness, springiness, gumminess, chewiness and resilience were recorded (Balasubramanian and Viswanathan 2007, Szczesniak 1985).

All the experiments were repeated for three times independently. The mean values for population density were transformed to logarithmic form and data were subjected to ANOVA and DMRT ($P \leq 0.05$) and TPA parameters were depicted graphically using Response Surface Plots.

Table 1 revealed that the increase in number of perforation accelerated the fungal population density and textural attributes of tempeh over non-perforated control. A further increment in all parameters was also noticed during the subsequent increase of incubation period up to 36 hr. The population density of tempeh was 1.5 log CFU/g at control and a maximum of 6.2 log CFU/g at 48 hr for 9 perforations. However, there were no significant differences between 48 hr 9 perforations-treatment and the treatments of 36 hr for 8 and 9 perforations ($P \leq 0.05$).

Highest textural value [viz. firmness (5.1 N), springiness (0.8), resilience (0.4), gumminess (3.1), chewiness (2.5)] was attained at 36 hr for 9 perforations that declined on extended incubation. Population density less than 5 log CFU/g was not adequate to bind the soybean grains together during 12 hr for all perforations and 24 hr for 0 and 7 perforations. Here, population of 6 log CFU/g at 36 hr incubation for 8 perforations was optimum for tempeh formation without spore or ammonia odour formation (Table 1, 2). The changes in population concentration and textural quality showed less variability in the appearance and pH of tempeh.

Fungal growth was directly proportional to the tempeh textural attributes up to 36 hr incubation only and was found inconsistent ahead. Loss in tensile strength of fungal mycelium by senescence with additional development of spores at extended incubation may be plausible explanation presently (Ariffin *et al*. 1994).

Table 1 Effect of perforation on *Rhizopous oryzae* growth at various incubation periods and comparison of fungal enumeration using most probable number (MPN) and spread plate (SP) method on acidified-potato dextrose medium (pH 3.5) on incubation at 30±2°C for 72 hr

Incubation period (hr)	Number of perforations	Population density[a] (log CFU/g)	
		MPN	SP
48	0	5.63±0.45 d	5.70±0.20 de
	7	6.07±0.06 ab	5.81±0.10 cd
	8	6.11±0.00 a	6.01±0.07 bcd
	9	6.15±0.07 a	6.34±0.14 a
36	0	5.74±0.09 cd	5.43±0.38 ef
	7	5.79±0.09 bcd	5.83±0.10 cd
	8	6.01±0.06 abc	6.12±0.31 abc
	9	6.11±0.06 a	6.28±0.22 ab
24	0	4.99±0.08 ef	4.79±0.10 g
	7	4.85±0.51 f	5.12±0.05 f
	8	5.30±0.03 e	5.22±0.05 f
	9	5.30±0.06 e	5.45±0.15 ef
12	0	4.18±0.06 g	3.87±0.09 j
	7	4.22±0.06 g	4.13±0.06 ij
	8	4.37±0.06 g	4.39±0.18 hi
	9	4.48±0.05 g	4.45±0.13 h
0	0	1.54±0.04 h	1.26±0.24 k
	7	1.61±0.10 h	1.46±0.15 k
	8	1.48±0.09 h	1.49±0.20 k
	9	1.45±0.06 h	1.50±0.19 k
cv (%)		3.63	3.92

[a]Mean values ± SD of three replicates are given. Means sharing a letter in the column are not significantly different ($P \leq 0.05$)

The effect of MPN method in respect to population density closely paralleled to spread plate method with coefficient of variation (*cv*) as 3.6 and 3.9 %, respectively. MPN assay in general resulted higher population count as compared to spread plate assay. However it's relatively low *cv* indicates higher reliability of the method (Gomez and Gomez 1984).

The results in this work clearly demonstrate that perforation numbers of container can significantly expedite the growth of *Rhizopus* mould and tempeh formation. This aspect offers scope for mathematically modelling the number of perforations to container sizes and growth of fungus to tempeh formation which is however underway in our lab to help the industry to reliably predict the optimum fungal population levels during processing. In addition it is evident from this work that MPN method can suitably replace the plate count method for fungal enumeration in tempeh research.

Table 2 pH values and appearance of tempeh during mycelial development.

Incubation period (hr)	Number of perforations	pH[a]	Characteristic of tempeh
48	0	7.7-8.4	Very dense mycelium; cake soft, without sponginess; very strong ammoniacal smell; some sporulation
	7	7.8-8.4	Very dense mycelium; cake soft, without sponginess; very strong ammoniacal smell; some sporulation
	8	7.9-8.0	Very dense mycelium; very firm brittle cake without sponginess; slight ammoniacal smell; some browning of mycelial strands
	9	7.7	Dense white mycelium; moderately firm but spongy cake
36	0	7.6-7.9	Very dense mycelium; very firm brittle cake without sponginess; slight ammoniacal smell; some browning of mycelial strands
	7	8.1	Very dense mycelium; very firm brittle cake without sponginess; slight ammoniacal smell; some browning of mycelial strands
	8	7.6	Dense white mycelium; moderately firm but spongy cake
	9	7.8	Dense white mycelium; moderately firm but spongy cake
24	0	6.7-7.0	Good mycelium; soft and fragile cake
	7	7.2	Good mycelium; soft and fragile cake
	8	6.6-7.3	Dense white mycelium; moderately firm but spongy cake
	9	6.7-7.3	Dense white mycelium; moderately firm but spongy cake
12	0	6.9	No visible hyphae
	7	6.7-6.9	No visible hyphae
	8	6.8-7.0	Slight mycelial growth
	9	6.8	Slight mycelial growth
0	0	6.6	Freshly inoculated cotyledons
	7	6.5-6.6	Freshly inoculated cotyledons
	8	6.4-6.7	Freshly inoculated cotyledons
	9	6.4	Freshly inoculated cotyledons

[a]Ranges of pH values for three replicates are given.

SUMMARY

A most-probable-number (MPN) method was adopted to determine the effect of perforation numbers (7, 8, 9) on fungal growth in tempeh in petriplate. The validity of the assay was verified using spread plate method. Preliminary observations reveal that perforation numbers significantly expedited the growth of fungus and well correlated with textural quality of tempeh at 0, 12, 24, 36 and 48 hr of incubation. Population density (6.1 log CFU/g) and textural magnitude [viz. firmness (5.1 N), springiness (0.8), resilience (0.4), gumminess (3.1), chewiness (2.5)] were significantly higher at 36 hr for 9 perforations than other treatments (P = 0.05).

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