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Optimization of Growth Conditions and Protease Activity for Tempeh Production from Sorghum

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Fermentation improves the nutraceutical properties of cereals. The objective of this work was to optimize the growth condition for tempeh production from sorghum. Tempeh was developed with sorghum and in combination with soybean. Effective proliferative growth of *Rhizopus oligosporus* (*R. oligosporus*) was found on all the substrates. The temperature had a significant effect on the growth of the mould, pH, and protease activity. The incubation temperature of 35^{O}C was found to be more favourable than 30^{o}C. The fermentation at 35^{o}C was completed within 36 hours compared to 30^{o}C (46 h). The pH was raised progressively up to 48 hours of incubation period (7.20-7.25). The protease activity at 35^{O}C was found to be increased until 36 hours and decreased thereafter. The protease activity was found to be significantly higher at 35^{o}C (99.20 IU /g) in comparison to 30^{o}C.

Keywords: Tempeh; protease; Rhizopus oligosporus; fermentation; soybean; sorghum.

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1. INTRODUCTION

In recent years, there is great difference in nutrition pattern and food preferences Nevertheless, interests for the consumption of the functional healthy foods have gained momentum due to consumer awareness [1,2]. Sorghum (*Sorghum bicolor*) known to us as Jowar, originated in Africa and has spread throughout the globe. Sorghum is a genus of about 25 species of flowering plants in the grass family Poaceae. Some of these species are grown as cereals for human consumption and some as pastures for animals. India ranks fifth in total sorghum production with 4.78 million tonnes grown in an area of 4.39 million hectares in 2020-21, Sorghum is generally consumed in India as traditional food forms mainly as porridge in rural and socioeconomically weaker section. Sorghum is widely used as forage for animals. Sorghum grain is a rich source of macronutrients (carbohydrates, proteins, and fat) and micronutrients (minerals and vitamins). It has about 70% carbohydrate, 3.5% fat and 11% protein. One of the major impediments for its use as food is the lower availability of protein, starch, and minerals due to the presence of anti-nutritional factors like tannins and phytic acid [3]. However, processing method like fermentation has proven to reduce the anti-nutritional factors, thus improving the nutritional availability and the functional properties of sorghum. Consumption of tempeh has been rapidly increasing nowadays, in many countries. Tempeh could be prepared with cereals and pulses other than soybean thereby increasing the acceptability and digestibility of the grains [2]. The intention of this study was to standardize the tempeh production at different temperatures from sorghum alone and in combination with soybean. The protease activity of the *Rhizopus oligosporus* culture was also studied.

2. MATERIALS AND METHODS

2.1 Materials

Tempeh starter culture *Rhizopus oligosporus* MTCC 556 strain was obtained from IMTECH. Chandigarh, the sorghum grains (*Sorghum bicolor*) and soybean (*Glycine max*) used in the study was procured from local market in Coimbatore. Chemicals used for the research were of analytical grade.

2.2 Inoculum Preparation

2.2.1 Preparation of *Rhizopus oligosporus* culture

The strain was grown on Potato dextrose agar (PDA) agar slant (Oxoid, CM0139, UK) for 48 h at 25^{o}C. The culture was then preserved at 4^{o}C and further sub cultured on the PDA slant at the interval of 30 days to retain the viability. Inoculum was prepared by transferring a 10 ml of sterilized distilled water to 48-hour old slant of *Rhizopus oligosporus.* The spores were taken under the sterile conditions and inoculum used in this study had a concentration of 10^{7} spores / ml [4].

2.2.2 Tempeh preparation

The tempeh was prepared in the laboratory according to the procedure described by [5] as follows: First, sorghum and soybean were cleaned to remove dirt, stones, weed seeds, damaged grains and any other extraneous matter. The grains were taken as per the experiment treatments such as T1-Soybean (control), T2- Soybean +Sorghum (1;1). T3-Soybean +Sorghum (1:2), T4-Soybean +Sorghum (1:3) and T5 – Sorghum. They were washed and soaked in clean tap water for 1 h at room temperature (28°C), and then boiled for 30 minutes to partially hydrate the grains. The hulls were then removed by hand, The grains were then soaked in water overnight and the excess hulls were removed, subsequently the grains were boiled for 60 minutes to facilitate partial cooking. Following the precooking the grains were cooled to 37^{o}C and air dried prior to inoculation. Before inoculation of fungal spore suspension, the pH of the substrate was adjusted to 5.5 using acetic acid at 2.0 mL per 100 g of substrate to maintain uniform pH in all substrates and to facilitate the growth of fungi. The grains were inoculated with *R. oligosporus* MTCC 556 @ 0,05 g per 100 g of distributed grains. The samples were mixed thoroughly such that the mould spore was evenly distributed over the surface of all grains. The grains were then packed in plastic bags perforated at 0,25 cm intervals and incubated at 30^{0} C and 35^{0}C and 40^{0}C,

2.2.3 pH measurement of fermented tempeh

The pH of the incubated tempeh at different temperature was monitored during fermentation at every 12 hours intervals to study the pH changes and to determine the optimum

fermentation time and temperature. The pH was measured at room temperature (20˚C -25˚C) using a digital pH meter. The pH meter was calibrated with buffer standards of pH 4.0 and pH7.0 before use. The measurements were taken in triplicate and average values was calculated.

2.3 Protease Assay

2.3.1 Crude enzyme extract

Tempeh (10 g) were mixed with 100 mL of 0.05 M phosphate buffer (pH 5) and homogenized. The homogenized mixture was kept at room temperature for 30 min with frequent stirring, and then centrifuged at 10000 rpm for 10 min. The supernatant was used as crude enzyme extract.

2.3.2 Protease activity

Protease activity was determined by the method of Yang and Huang [6]. The reaction mixture containing 2 mL of 1% casein solution in 0.05 M phosphate buffer (pH 5) and 1 mL of enzyme solution were incubated at 60^{o}C for 15 min and the reaction was then stopped with the addition of 3 mL of 10% trichloroacetic acid. After 10 min the entire mixture was centrifuged at 10000 rpm for 10 min at 4^{o}C and the absorbance of the liberated tyrosine in the filtrate was measured at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1µg of tyrosine per min under assay conditions.

2.4 Statistical Analysis

Data were assessed by analysis of variance (ANOVA) using SPSS program version 16.0 and means were separated by Duncan's multiple range test with a probability ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Effect of Temperature on Growth Characteristics and Duration of Fermentation

Growth of *R. oligosporus* on the substrates incubated at different temperatures is represented in Fig. 1. The results indicated difference among temperature ranges (30, 35 and 40^{o}C). The favourable temperature for the growth of *R. oligosporus* was found to be 30 and 35^{o} C, while no growth was observed at 40^{o}C indicating that 30 and 35^{o}C were found to be the optimum temperatures for fermentation of sorghum tempeh. The temperature within the fermenting beans mass does not raise above approximately 40^{o}C or the high temperature may damage subsequent growth of the mould [7]. Incubation temperature of 37^{o}C favoured the growth of *R. oligosporus* while being less favourable for growth of the mesophilic moulds and fewer bacterial species [8]. There was significant difference among the temperature on growth of *Rhizopus oligosporus.* The fermentation was significantly superior and quicker at 35^{O}C than 30^{o}C in all treatments. Among the substrates T1(soybean 100%) and T2 (Soybean+Sorghum 1:1) recorded less fermentation duration of 36 hours while in T5(sorghum 100%) the duration for complete growth of the *Rhizopus* was found to be 38 hours at 35^{o}C. The fermentation duration ranged from 46 to 49 hours at 30^{O}C as depicted in Fig. 1. The best temperature for tempeh production from sorghum alone and in combination with soybean was found to be 35^{o}C. This corroborated well with findings of Han et al., [9] and Reddy et al., [10]. The optimum temperature of 35.8^{o}C was required for chickpea tempeh formation with *Rhizopus stolonifera* [11].

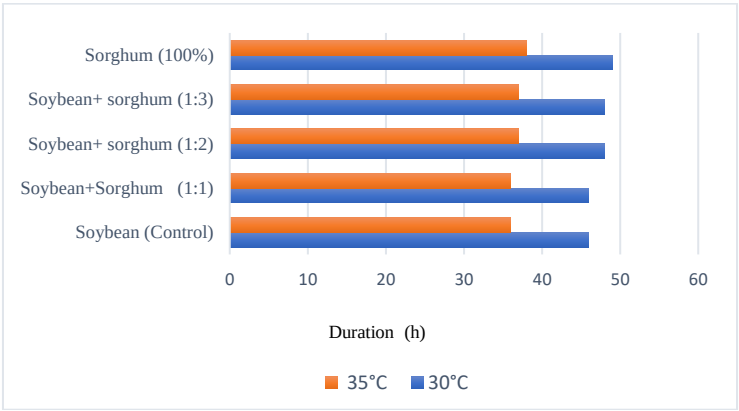

Fig. 1. Effect of temperature on tempeh fermentation

Table 1. Effect of temperature on pH during tempeh fermentation at different time intervals

Treatments	Temperature ^{0}C	Fermentation Time (hours)				
		12	24	36	48	60
Soybean (100%)	30	6.10± 0.02^{cA}	6.30±0.06^{dB}	6.50±0.04^{cC}	6.90±0.02^{bD}	7.20±0.02^{aE}
	35	6.10±0.02^{bA}	6.35±0.0^{cB}	6.60±0.06^{eC}	7.20±0.00^{cD}	-
Soybean + Sorghum (1:1)	30	5.90±0.03^{aA}	6,27±0.04^{bB}	6.55±0.02^{cC}	6.90±0.02^{bD}	7.30±0.04^{aE}
	35	6.00±0.04^{bA}	6.38±0.02^{eB}	6.55±0.04^{cC}	7.20±0.04^{cd}	-
Soybean+ Sorghum (1:2)	30	5.80±0.04^{aA}	6.12±0.04^{aB}	6,38±0.02^{bC}	6.90±0.04^{bD}	7.30±0.02^{aE}
	35	5.90±0.04^{aA}	6.24±0.04 ^{B}	6.50±0.06^{cC}	7.25±0.04^{cD}	-
Soybean+ Sorghum (1:3)	30	5.75±0.04^{aA}	5.90±0.02^{aB}	6.20±0.04^{aC}	6.75±0.03^{aD}	7.25±0.02^{aE}
	35	5.80±0.02^{aA}	6.10±0.06^{aB}	6.55±0.06^{cC}	7.20±0.04^{cD}	-
Sorghum (100%)	30	5.75±0.04^{aA}	6.20±0.02^{bB}	6,45±0.04^{bC}	6.80±0.04^{aD}	7.30±0.02^{aE}
	35	5,80±0.06^{aA}	6,25±0.04^{bB}	6.50±0.06^{cC}	7.25±0.02^{cD}	-

Values are mean±standard deviations of triplicates.
Means with small alphabet superscripts within columns, and those with capital alphabet superscripts within rows are significantly different ($p < 0.05$)

Table 2. Effect of temperature on protease activity (IU/g) during fermentation at different time intervals

Treatments	Temperature ^{0}C	Fermentation Time (hours)				
		12	24	36	48	60
Soybean (100%)	30	7.04±0.02^{iA}	76.08±0.04^{gB}	92.65±0.04^{fC}	93.20±0.02^{gD}	8.54±0.04^{dE}
	35	7.58±0.04^{JA}	84.38±0.02^{hB}	99.20±0.04^{gC}	9.74±0.04^{cD}	-
Soybean + Sorghum (1:1)	30	5.84±0.02^{gA}	72.80±0.04^{eB}	86.20±0.04^{Dc}	88.27±0.05^{fD}	4.60±0.02^{c}
	35	6.34±0.04^{hA}	75.10±0.04^{fB}	90.32±0.02^{ec}	8.26±0.02^{bD}	-
Soybean + Sorghum (1:2)	30	4.08±0.03^{dA}	69.50±0.02^{cB}	80.90±0.04^{bc}	82.34±0.04^{eD}	4.80±0.02^{cE}
	35	4,78±0.02^{fA}	73.20±0.02^{eB}	85.50±0.06^{dc}	8.20±0.04^{bD}	4.40±0.03^{bE}
Soybean + Sorghum (1:3)	30	3,72±0.00^{cA}	65.56±0.03^{bB}	77.25±0.05^{ac}	79.56±0.04^{dD}	4.20±0.00^{bE}
	35	4.53±0.02^{eA}	71,80±0.04^{dB}	83.60±0.05^{cc}	7.87±0.04^{bD}	4.60±0.02^{cE}
Sorghum (100%)	30	2.52±0.04^{aA}	63.21±0.04^{aB}	76.50±0.04^{ac}	78.13±0.02^{dD}	2.19±0.04^{aE}
	35	2.86±0.02^{bA}	71,20±0.05^{dB}	83.24±0.06^{cc}	4.88±0.04^{aD}	-

Values are mean±standard deviations of triplicates.Means with small alphabet superscripts within columns, and those with capital alphabet superscripts within rows are significantly different ($p < 0.05$)

3.2 Changes in pH

Table 1 reveals the effect of temperature on pH of the substrates during fermentation at different time intervals. Growth of the mould was accompanied by rapid increase in pH in all the treatments. The pH increased up to 48 hours (7.20-7.25) and remained constant thereafter at incubation temperature of 35^{o}C in all the treatments. At 30^{o}C the pH increased steadily up to 60 hours (7,20-7,30) beyond which it remained constant. The increase in pH value is due to the significances of protein metabolism by the moulds. Muzdalifah et al. [12] reported that the longer the tempeh fermentation time, the increase in pH exceeded 8. R.A. Sparringa, and J.D. Owens,[4] stated that pH increased to 6.6 - 7.1 in matured tempeh as result of ammonia production.

3.3 Protease Activity

The protease activity during tempeh fermentation of sorghum alone and in combination with soybean incubated at different time intervals are presented in Table 2. Protease activity increased with increase in duration of fermentation up to 36 hours beyond which it decreased significantly. The protease activity was significantly highest at 35^{o}C when compared to 30^{o}C.Protease activity was maximum and significantly higher in Soybean 100% (99.20 IU/g) followed by Soybean+Sorghum (1::1) treatment (90.32 IU /g). Least protease activity of 83.2 IU/g was observed in sorghum 100%. Muhammad Gul Sher et al. [13] reported the maximum protease units (99.52 + 1.12 IU/g) in fermented barley at 36 hrs of incubation.

4. CONCLUSION

The present preliminary study, demonstrated that tempeh could be developed from sorghum alone and in combination with soybean. The optimum temperature for proliferative growth of *R. oligosporus* and maximum protease production was standardised as 35^{O}C.Thus fermentation could open an avenue for better utilisation of neglected cereal like sorghum for human nutrition.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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