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Fermentative Production of White Pepper Using Indigenous Bacterial Isolates

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Abstract Three *Bacillus* strains were isolated from soil samples. Morphological and physiological characterization indicated that the isolated strains were *B. mycoides*, *B. licheniformis* and *B. brevis*. White pepper was produced from black pepper by the fermentative method using the isolates in shake flaks as well as in a large-scale fermenter. Volatile oil and piperine contents of the product were 3.2% (v/w) and 4% (v/w) respectively. The moisture content was 15%. The microbial contamination was less than 10 per 100 g. The product also exhibited excellent storage stability.

Keywords: white pepper, retting, bacteria, *Bacillus*, fermentation

INTRODUCTION

White pepper is a value-added form of black pepper (*Piper nigrum* L.). It possesses a mild flavor and pungency as compared to black pepper, which has a sharp, pungent aroma and flavor [1]. Due to its mild flavour, pungency and light color, there is a growing demand for white pepper in the markets worldwide. To produce white pepper traditionally, the pepper berries are allowed to ripen more fully on the vine. Fully ripened cherry-colored berries are separated from the stalk, tightly packed into jute bags and steeped in slow-running water for about 2~3 weeks. During this process of retting, the outer skin (pericarp) is decayed and loosened from the core of the fruit. The bags are then trampled on until the pericarp is removed, which is then rubbed off in water. The white pepper cores obtained are washed several times and sun dried for two to three days. In the traditional process, ripe and immature berries are often used and results in a mixture of black and white cores. Sorting of the white pepper cores is a time-consuming and labor-intensive process that increases the labor cost. In addition, this method also leads to the risk of heavy microbial contamination. Furthermore, the farmers also entail huge loss of berries due to birds feeding on ripe red berries used for the production of white pepper.

It may also be produced by other methods like steaming and mechanical decortication. However, white pepper produced by these methods does not yield the same aroma and flavour in contrast to that obtained by traditional methods [2]. Another alternative is to use microbial methods for the production of white pepper. However, there is no published evidence for the use of microbial strains for the retting of black pepper. The present study investigates the use of bacterial isolates for the fermentative production of white pepper.

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MATERIALS AND METHODS

Materials

Commercial black pepper of uniform grade and size used in this study was purchased from the local market. Nutrient broth was obtained from HiMedia Laboratories, Mumbai, India. All other chemicals used were of analytical grade.

Microorganism and Maintenance

Three isolates of *Bacillus* strains from soil samples were examined for morphological, physiological and biochemical characteristics identified as per standard methods [3] and used in the retting of black pepper. The isolates were maintained on nutrient agar plates containing (% w/v): peptone 2, yeast extract 0.5, beef extract 1.0, sodium chloride 0.5, agar 3 and pH 7.2 to 7.4.

Shake Flask Fermentations

The bacterial strains were cultivated in 100 mL of nutrient broth of varying strengths (0.23, 1.15 and 2.3% w/v) and at pH 7.0~7.2 in 500-mL Erlenmeyer flasks. The flasks were inoculated in triplicate with a 6 h culture of each isolate. 10, 20, 30, and 50 g of black pepper were added into each set of the three flasks. The flasks were incubated at 37°C in a rotary shaker at 80 rpm. Flasks were examined at every 12 h for growth as well as for

Table 1. Morphological and physiological characteristics of the bacterial isolates

Characteristics	*B. mycoides*	*B. licheniformis*	*B. brevis*
Shape	Narrow capsule	Narrow capsule	Narrow capsule
Gram staining	+	+	+
Motility of single cells	Sluggishly motile	Sluggishly motile	Sluggishly motile
Formation of spore	–	–	–
Catalase	–	+	+
Oxidase	–	+	+
Voges-Proskauer test	–	+	+
Acid formation from glucose	+	+	+
Arabinose	–	–	+
Xylose	–	–	+
Mannitol	–	–	+
Gas formation from glucose	–	–	–
Degradation of casein	+	+	+
Liquefaction of gelatin	+	+	+
Citrate	+	+	+
Nitrate reduction	+	+	+
Formation of indole	–	–	–
Degradation of starch	+	+	+
Utilization of cellulose	–	–	+
Cellobiose	–	–	+
Genera	*B. mycoides*	*B. licheniformis*	*B. brevis*

loosening of the pericarp of the berries. After 96 h, the culture supernatants from the flasks were decanted and the white pepper cores obtained were washed repeatedly in water and dried in shady sun.

Large-scale Fermentation

Large-scale fermentations were carried out using the mixed culture of the three isolates in an indigenously made 50-L stainless steel fermenter equipped with an agitator and a thermostat for temperature control. The growth medium consisted of 25 L of 0.5 N nutrient broth at pH 7.2 prepared using ordinary water. The contents were *in situ* sterilized for 30 min and cooled to 35°C (optimum temperature for maximal rate of growth of the isolates). The growth was initiated by the addition of 3 L of the preculture prepared by incubating the bacterial isolates separately in 1 L of nutrient broth at pH 7~7.5 in shake flasks at 37°C and 80 to 100 rpm for 6 h. 10 kg of the commercial black pepper berries was added into the reactor without any pretreatment or washing. The contents were mixed only once in 24 h. Temperature was initially set at 35° C and controlled for 24 h. Heating was discontinued afterwards. After 12 days, the white pepper cores were collected after draining the culture supernatant from the reactor, washed several times in water and dried in shaded sun for about 8 h.

Analytical Methods

White pepper was analyzed for moisture content, total microbial count (plate count), and coliform count (MPN) as per the standard methods [4]. Total microbial count was determined by the spread plate technique on nutrient agar. 10 g of the sample was suspended in sterile saline and 0.1 mL of the solution was spread on nutrient agar. After incubation at 37°C, the developed colonies were counted. The volatile oil content in the white pepper was estimated by the method 5.0 of American Spice Trade Association [5]. Soxhlet extraction with petroleum ether was carried out to determine the piperine content. The biomass was monitored by the viable cell count.

RESULTS AND DISCUSSION

Identification of the Isolates

The isolated strains were found to posses the properties listed in Table 1. In summary, the strains were non-spore forming, gram-positive aerobes with sluggish motility. They were identified as *B. mycoides*, *B. licheniformis* and *B. brevis*. Characterization of the enzymatic activities revealed that all the three isolates were capable of synthesizing extracellular amylases, proteases and cel-

Table 2. The time course of biomass and the percentage of skin removal during the fermentative production of white pepper using the three bacterial isolates in shake flasks

Fermentation time (h)	Isolates					
	B. mycoides		*B. licheniformis*		*B. brevis*	
	Biomass (g/L)	Skin removed (%)	Biomass (g/L)	Skin removed (%)	Biomass (g/L)	Skin removed (%)
12	0.2	*	*	*	0.5	*
24	1.4	15	0.5	15	1.9	5.0
48	2.9	40	0.9	60	2.8	50
72	3.4	80	1.2	80	6.0	75
96	3.3	80	1.3	80	8.0	95~98

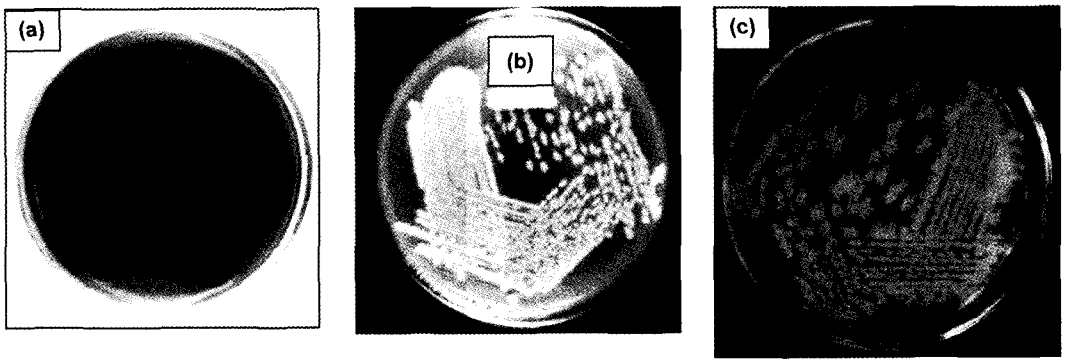

Fig. 1. Growth of (a) *B. mycoides* on carboxymethylcellulose agar, (b) *B. licheniformis* on starch agar, and (c) *B. brevis* on casein agar indicating the production of cellulase, amylase, and protease, respectively.

lulases when tested with substrates like starch, casein, gelatin, cellulose and cellobiose in agar medium. Concerning their ability to use sugars as carbon sources, all isolates showed quite homogenous behavior with regard to glucose. While *B. mycoides* and *B. licheniformis* lacked the ability to use arabinose, xylose and mannitol, *B. brevis* was able to utilize theses sugars. All the isolates utilized citrate as carbon source. While *B. licheniformis* and *B. brevis* produced catalase and oxidase, *B. mycoides* was unable to produce theses enzymes. With regard to the temperature dependence of growth, all the isolates grew at 30 and 40°C. There was no growth below this temperature. While *B. mycoides* grew at 50 and 55°C, *B. licheniformis* could not grow above 40°C. *B. brevis* was found to be thermophilic. All the three strains exhibited good growth at pH 5.7 and 6.8.

Shake Flask Studies

Production of white pepper was carried out using each of the isolated strains as well as the mixed culture in shake flasks. The optimum time for maximal growth rate and secretion of enzymes, which caused the retting of black pepper, varied widely with individual strains. The time course of biomass and the percentage of skin removal by the three bacterial isolates in shake flasks are given in Table 2. Nearly 80% deskinning was achieved with *B. mycoides* and *B. licheniformis* in 48 to 72 h when the biomass density reached 3.4 and 1.2 g/L respectively. Beyond 72 h, further deskinning was found to be slow. In the case of *B. brevis*, loosening of the outer skin started after 24 h and 90% of the skin was removed in 96 h. *B. brevis* also showed vigorous growth in 0.1 N and 0.5 N nutrient broths leading to a high biomass concentration of 6 to 8 g/L in 96 h. As far as retting is concerned, the earliest change was observed in *B. brevis* cultures within about 12 h even at 30% pepper concentration, while there was slow but sustained digestion by *B. mycoides* and *B. licheniformis*.

When a mixture of the three isolates was used in shake flasks, the performance was at its best with very early and sustained retting. Retting was first noticed within 18 h of inoculation and nearly 95 to 98% of the deskinning of black pepper was completed in 96 h in shake flask cultures. Fig. 2 (a) and (b) show the pepper berries before and after the retting process in shake flasks using the mixed culture. The berries were creamy white, firm, striated, and retained the thin membranous covering.

The bacterial isolates synthesized and secreted hydrolytic enzymes like cellulase, amylase, and protease [6]. The presence of these exoenzymes was confirmed by the appearance of clear areas on starch-agar, carboxymethylcellulose-agar and casein-agar plates (Fig. 1). These enzymes, which were produced during the bacterial growth,

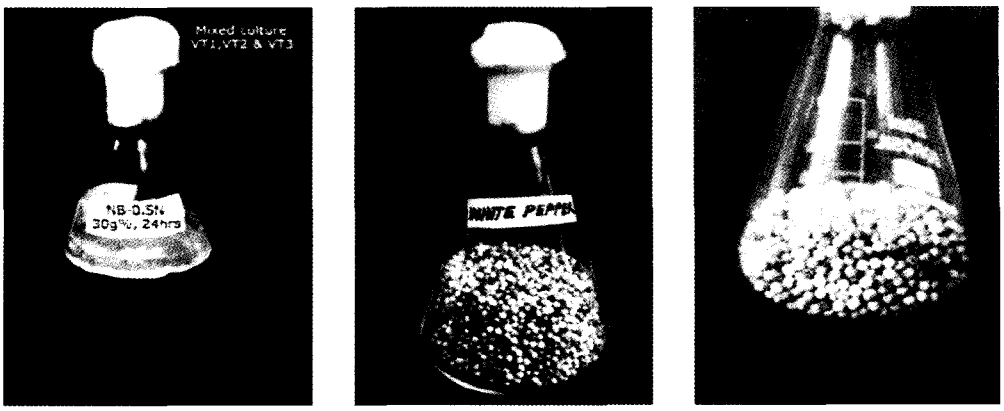

Fig. 2. (a) Bacterial retting of black pepper berries (30% w/v) by the mixed culture of *B. mycoides*, *B. licheniformis* and *B. brevis* in 0.23% (w/v) normal nutrient broth at pH 7.0~7.2 and 37°C, (b) white pepper produced in shake flask fermentations by the mixed culture, and (c) white pepper produced in 50-L fermenter by the mixed culture.

Table 3. Comparison of the properties of various forms of pepper

Material	Moisture content (%)	Microbial count (per 100 g)	Volatile oils (%)	Oleoresins (%)
Black pepper	15	10^5	3.6	4.2
White pepper	15	10	3.2	4.0
Commercial white pepper	12	10^{14}	1.0	2.0

broke down the respective macromolecules present in the epicarp and pericarp of the black pepper berries. The skin was thus detached and dissolved away resulting in the formation of white pepper cores.

Large-scale Fermentations

Large-scale production of white pepper was attempted in the indigenous fermenter and using the mixed culture of the three isolates. Since very early and sustained retting was obtained using the mixed culture of the three isolates, it was decided to implement large-scale fermentations with the mixed culture. The contents of the reactor became turbid with intense surface growth in 48 h and the pericarp of the black pepper berries started to loosen. Growth reached stationary phase at 96 h and the rate of retting decelerated with further increase in time. Nutrients were replenished by the addition of glucose at a concentration of 0.1% (w/v) every 24 h. More than 90-95% of pepper turned white in 10~12 days. Fig. 2 (c) shows a sample of white pepper from 50-L reactor with similar product quality as that obtained from the shake flasks (Fig. 2(b)). The broth remained aromatic with a characteristic flavour and pungent odor. Since the removal of the skin was closely associated with the growth, the enzymes involved were synthesized as a result of primary metabolism.

Quality and Yield of White Pepper

Table 3 shows the comparison of the properties of the white pepper produced by the fermentative method using the bacterial isolates with that of black pepper and commercial white pepper. The moisture content of the product was comparable with that of black pepper. The moisture content of the white pepper was 15%, while the moisture contents of the black pepper and the commercial white pepper were 15% and 12% respectively. Volatile oil and piperine content in the product were 3.2 and 4% (v/w) as compared to 3.6 and 4.2% (v/w) in black pepper. The volatile oil and piperine contents of the commercial white pepper were only 1% and 2% (v/w) respectively. When comparing the quality of a commercial product, the shelf life is an important factor. At ambient temperatures varying between 30~34°C, 50~60% of volatile oils and oleoresins were retained after 8 years. The products were also stored under refrigerated conditions in order to find out whether storage under lower temperature is essential for the retention of aroma and pungency and to evaluate the extent of influence. White pepper produced by bacterial fermentation retained more than 80% of aroma and volatile oils when stored in a refrigerator for 8 years. The aroma and flavour are attributed to the volatile oils and alkaloids present in the white pepper. These are retained markedly in our process of retting. White pepper available in the market was found to contain only very low amounts of theses constituents (Table 3). Mechanical decortications, one of the conventional methods to produce white pepper, often leads to great loss of active principles. Soaking of black pepper in gunny bags in stagnant water or rivers for the natural retting, multiple microbes decay skin under varying con-

ditions resulting in the development of bad odours and loss of volatile oils and oleoresins.

The most significant feature of white pepper produced by the fermentative method was its extremely low microbial contamination. The total microbial count was less than 10 per 100 g of white pepper (Table 3). The commercial white pepper produced by the conventional method had a very high microbial count of 10^{14}. Carrying out the retting in laboratory controlled hygienic sterile conditions, extensive washing of the white pepper in good quality water, followed by sun drying for about 8 h has resulted in a superior quality product with very low microbial count as compared to what is available in the market, prepared by the conventional retting of ripe berries in open streams or rivers. Furthermore, decreased microbial count in the final product would enhance the shelf life significantly. The yield of white pepper from the fermentative method was 90 to 98% based on the amount of black pepper initially used. The creamy white pepper cores also retained its color without any microbial contamination.

White pepper is a major food item, which is highly contaminated with molds, yeasts and bacteria [7]. Several methods like fumigation with ethylene oxide, treatment with superheated steam and exposure to ionizing radiations are commonly employed on a commercial basis for the decontamination of white pepper. While sanitization by fumigation is accompanied by many problems like decrease in volatile compounds, toxicity to applicators and flammability during treatment [8], treatment with ionizing radiations can lead to degradation of polysaccharides, such as starch in addition to insufficient public acceptance. Fermentative production of white pepper using the bacterial isolates is therefore a more suitable method due to exceptionally low level of microbial load and superior quality as compared to that produced by the traditional retting process.

CONCLUSION

There is a growing demand for white pepper owing to its mild flavor and pungency as compared to black pepper. It is produced by the deskinning of black pepper berries. The traditional method of deskinning by retting in water followed by sun drying results in inferior quality product with high microbial contamination, besides being highly labour oriented. This study made clear that the retting method has an influence on the quality of white pepper. The fermentative method of production of white pepper demonstrated a greater capability in providing a higher yield of superior quality white pepper at a relatively short time. The volatile oil and piperine contents, which are responsible for the aroma, were conspicuously enhanced in the fermentatively produced white pepper. Besides, the product had a lower microbial load as compared to that of the commercial white pepper. Collectively, these results illustrated the competitiveness of the fermentative method for the industrial-scale production of excellent quality white pepper.

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