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ORIGINAL ARTICLE

Isolation, characterization and identification of pericarp-degrading bacteria for the production of off-odour-free white pepper from fresh berries of *Piper nigrum* L.

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Keywords
16S rDNA, *Bacillus*, black pepper, oleoresin, skatole, white pepper.

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Abstract

Aim: To isolate, fermentatively evaluate and identify black pepper (*Piper nigrum* L.)-associated bacteria for the microbial decortication of fresh ripened berries and dried black pepper for preparation of off-odour-free white pepper.
Methods and Results: Among 45 bacterial isolates obtained from black pepper, seven of them were found to decorticate black pepper (>60%) and fresh pepper berries (98–100%) into white pepper within 5 days of immersion in bacterial suspension. The 16S rRNA genes (1500-bp amplicon) of these bacteria were sequenced, and species identity was established by closest match in GenBank. Superior-quality white pepper was obtained with *Bacillus subtilis* (IISR WP 33, 34, 38), *Bacillus licheniformis* (IISR WP 43), *Acinetobacter baumanii* (IISR WP 35), *Klebsiella pneumoniae* (IISR WP 19) and *Microbacterium barkeri* (IISR WP25). The bacterial isolates were found to secrete multiple hydrolytic enzymes such as cellulase, pectinase, amylase, protease and xylanase. Bacterial cultures were deposited with International Depository Authority at Microbial Type Culture Collection, India, as patent deposits as prescribed in Budapest Treaty for microbial deposits. The white pepper, thus obtained from bacterial decortication process, was free from off-odour compound, especially skatole. Other biochemical constituents such as oleoresin, piperine and essential oils were found in the acceptable range. The bacterial decortication did not affect inherent constituents of pepper such as essential oil constituents, oleoresin and piperine content.
Conclusion: One of the most significant findings of the work is identification of specific bacterial species for decortication of fresh berries or black pepper berries into value-added white pepper.
Significance and Impact of the Study: This work paved way for developing a technological process for microbial decortication of fresh/black pepper for the production of superior-quality white pepper.

Introduction

White pepper is one of the value-added forms of black pepper that fetches a high value owing to superior quality and suitability to use in wide range of food materials and low microbial contaminants. Indonesia is the largest white pepper producing country, which converts about half of its pepper to white. White pepper is produced by the decortication of ripened pepper berries or the dried black pepper. The traditional retting method (Natarajan *et al.* 1967; Lewis *et al.* 1968; Madhusoodanan *et al.* 1990), mechanical decortications (Thomas *et al.* 1987), chemical methods (Joshi 1962; Lewis 1982) and enzymatic decortication (Gopinathan and Manilal 2004) are

inadequate for the bulk production of white pepper. The traditional systems for white pepper preparation encounter issues such as the unacceptable production method and inferior quality of end product. Pepper berries soaked in natural stream lead to contamination by micro-organisms or toxic wastes which lead to swampy odour on white pepper (Jagella and Grosch 1999; Steinhaus and Schieberle 2005). Targeted fermentation using known microbial species can be exploited for the preparation of white pepper. Microbes are known to secrete several hydrolytic enzymes, which are capable of degrading the components of pericarp (Thankamony *et al.* 1999; Gopinathan and Manilal 2005). Inherent quality of white pepper should not be compromised during the fermentative decortication process. More specifically, the berries with acceptable level of essential oil, oleoresin, piperine, colour, texture and odour are among the critical factors that influence the international white pepper trade. Among the other quality parameters, complete absence of off-odour compound, skatole, the by-product of bacterial interaction on raw berries, is an issue which decides the consumer acceptance and trade. White pepper with a pronounced faecal off-odour is due to the accumulation of 3-methyl indole (skatole) and 4 methyl phenol (p-cresol). Additional odorants with offensive odour were identified as 3-methylphenol, butanoic acid and 2-/3-methyl butanoic acid. These compounds are not genuine pepper compounds which are accumulated on the berries during fermentation (Steinhaus and Schieberle 2005). Major objectives of our work include isolation and identification of bacterial flora from black pepper for their exploitation as microbial agents in decorticating (deskinning) fresh berries or dried black pepper into value-added white pepper. We have further analysed several physical and biochemical quality parameters, especially the skatole in white pepper prepared by targeted bacterial fermentation of matured and fresh ripened berries.

Materials and methods

Isolation of bacteria

Bacteria were isolated from black pepper-associated sources such as black pepper berries, water-soaked black pepper berries, decomposed berries and commercially available white pepper on glucose potassium nitrate medium (g l^{-1}) (Glucose – 5, KNO_3 – 3·5, KH_2PO_4 – 1·75, $MgSO_4.H_2O$ – 0·75 and Agar-18, pH-7·2), where glucose is substituted by various carbon sources (5 g l^{-1}) such as citrus pectin, or carboxy methylcellulose (CMC), or finely powdered black pepper pericarp or starch by pour plate method (Table S1). One gram of each of black pepper-associated sources was aseptically added to 30 ml phosphate buffer saline (PBS) (g l^{-1}) (NaCl – 8, KCl – 0·2, Na_2HPO_4 – 1·44 and KH_2PO_4 – 0·24, pH-7·4) and centrifuged at 1000 ***g*** at 4°C for a minute. The supernatant, thus obtained, was serially diluted up to 10^{-3}, pour plated on to above media and incubated at 25, 35 and 45°C for 2–3 days. Colonies of bacteria isolated on various media were glycerol (30%)-preserved at −80°C for further work.

Screening of bacteria for decortication of berries

Preliminary screening for pericarp decortication was conducted on black pepper. Luria Bertani broth (Hi Media, Mumbai, India) supplemented with either pectin (0·1% w/v), or CMC (0·1% w/v) or starch (0·1% w/v) was used as fermenting media at temperature regimes such as 25, 35 and 45°C. The experiment was conducted in completely randomized design (CRD) with replications. One hundred gram each of two forms of black pepper such as dried black pepper and boiled black pepper (at 100°C for 3 min) were used as raw material. After 5 days of fermentation, the pepper berries were gently pressed to remove pericarp, washed repeatedly and dried directly under sunlight for 3 days. Efficiency of bacteria for pericarp degradation is expressed as percentage decortication (Decortication %) and percentage recovery (Recovery %). Percentage decortication is the ratio between white pepper obtained and total berries fermented by count, whereas percentage recovery is ratio between white pepper obtained and total berries fermented by weight. The data were normalized by angular transformation and statistically analysed using MstatC program (www.msu.edu/~freed/disks.htm).

Phenotypic characterization

Isolates were grouped based on phenotypic characteristics such as colony colour, size, form, elevation, margin, surface, opacity and texture on nutrient agar. Other characters such as Gram reaction, motility, cell morphology and spore formation were also recorded. Routine biochemical tests such as indole; methyl red; Voges–Proskauer; citrate; the presence of oxidase and catalase; carbon utilization, hydrolysis of pectin, CMC, xylan, casein and starch; growth at 20, 30, 40, 50, 60, 70, 80°C; and growth at 1, 2, 5, 7 and 10% salt concentration were assessed for each bacteria, as described by Zvyagintsev (1991).

Identification by sequencing of 16S rDNA

Genomic DNA from the bacteria was isolated according to the protocol of Kumar *et al.* (2004). Amplification of 16S rRNA gene was performed on Master Cycler

Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany), with universal primer set pA (FP) (5′-AGA GTT TGA TCC TGG CTC AG- 3′) and pH (RP) (5′-AAG GAG GTG ATC CAG CCG CA- 3′) (Woese 1987; Stackebrandt and Goebel 1994) in 25 μl of reaction mixture containing 1X *Taq* buffer, 100 μmol l^{-1} dNTPs mix, 3 mmol l^{-1} $MgCl_2$, 10 μg BSA, 10 pMol each primer, 0·5 U of *Taq* DNA polymerase and 100 ng template DNA. The thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min, 35 amplification cycles of 94°C for 1 min 10 s, 48°C for 30 s, 72°C for 2 min 10 s and a final polymerization step of 72°C for 6 min 10 s. The final PCR product was resolved in 1% agarose gel, excised and purified with elution kit (Sigma-Aldrich, St Louis, MO, USA). The cycle sequencing reaction was performed with 20–30 ng of purified amplicon using the ABI PRISM BigDye Terminators v1·1 cycle sequencing kit according to the manufacturer's instruction (Applied Biosystems, Foster city, CA). The purified product was sequenced bidirectionally to obtain complete coverage of the gene. The sequences were edited, contig assembled in CLC Bio Sequence viewer, compared with GenBank sequences by BLAST analysis and accession number assigned. Nucleotide sequence similarities were determined using the NCBI or EMBL databases, and sequence identity *vis-à-vis* the bacterial identity was established by closest match (Altschul *et al.* 1990).

Evaluation of shortlisted bacteria for decortication of berries and culture deposition

Having identified in preliminary screen, the seven shortlisted bacteria viz., *Bacillus subtilis* (three isolates), *Bacillus licheniformis* (one isolate), *Microbacterium barkeri* (one isolate), *Klebsiella pneumoniae* (one isolate) and *Acinetobacter baumanii* (one isolate) were subjected to further evaluation on partially ripened and freshly harvested berries for preparation of white pepper. Three litres of the 24-h-old bacterial culture (1·5–2·0 OD at 600 nm) was diluted with sterile water (1 : 1) and added to thoroughly washed fresh berries (15 kg). Uninoculated media served as control. The pepper berries were incubated at 35°C for 5 days. Temperature and pH of the medium was recorded regularly. After 5 days of incubation, the pepper berries were trampled and thoroughly washed with tap water to remove degraded pericarp and bacteria. The creamy white pepper berries, thus, obtained from the fermentation was dried under sunlight for 3 days and stored in the sterile containers. Percentage decortication was estimated for each of the bacterial isolate in a parallel experiment that involved a sample of 100 berries replicated thrice. Percentage recovery was estimated as described above. Five among the promising bacteria were deposited as patent deposits, with International Depository Authority at Microbial Type Culture Collection (MTCC), as prescribed in Budapest Treaty for microbial deposition.

Assay for hydrolytic enzymes

The shortlisted bacteria were assayed for hydrolytic enzymes such as CMCase (Chen *et al.* 2004); xylanase assay (Gupta *et al.* 2009); pectinase assay (Soares *et al.* 1999); amylase assay (Dubey and Maheshwari 2002); and protease assay (Dubey and Maheshwari 2002). On the agar plates, wells were made using the aseptic cork borer and filled with 0·1 ml of bacterial cell suspension in sterile water, having an absorbance of 0·1 OD units at 600 nm. Sterile distilled water served as mock. The plates were incubated at 35°C for 2 days and examined by staining for a halo around the well which indicated the production of extracellular enzymes.

Estimation of physical and biochemical parameters of white pepper

Physical and biochemical quality parameters of microbially decorticated white pepper were assayed by adopting methodologies suggested by American Spice Trade Association (ASTA). The moisture content of white pepper was analysed using HG53 Halogen moisture analyzer (Mettler Toledo, Columbus, OH, USA). Bulk density is measured by the weight of the white pepper per unit volume (ASTA 1968). The starch was extracted and estimated with perchloric acid method (Sadasivam and Manickam 1992). Oleoresin content was estimated by standard acetone percolation method of ASTA (1968). Piperine content was determined by HPLC analysis (Shimadzu, Kyoto, Japan). Standard piperine solution was prepared by the addition of 2 ml of piperine solution (1 mg ml^{-1}) and 2 ml of internal standard solution (0·5 mg ml^{-1} of *p*-dimethylamino benzaldehyde) in a 25-ml volumetric flask, and final volume was made up to 25 ml with methanol. The column used was C 18 (25 cm × 4·6 mm) with mobile phase 1% acetonitrile: acetic acid, (1 : 1) and a flow rate of 1·5 ml min^{-1}.The essential oil was analysed using a Shimadzu GC-2010 gas chromatograph equipped with QP 2010 mass spectrometer (Zachariah *et al.* 2010). RTX-5 column (30 m × 0·25 mm, film thickness 0·25 μm) was used. Helium was used as the carrier gas at a flow rate of 1·67 ml min^{-1}. The injection port was maintained at 250°C; the detector temperature was 220°C; oven temperature was programmed as follows: 60°C for 5 min and then increased to 110°C at the rate of 5°C min^{-1}, then up to 170°C at the rate of 3°C min^{-1}, again up to 220°C at the rate of 5°C min^{-1}, at which the column was

maintained for 3 min. The split ratio was 1 : 40 and ionization energy 70 eV. The retention indices were calculated relative to C8–C20 n-alkanes. The constituents of the oil were identified by a comparison of retention indices with those reported in literature, by matching the mass spectral data with those stored in NIST and Wiley library and wherever possible, by co-injection with authentic standards.

Estimation of off-odour compounds in white pepper

White pepper obtained from microbial fermentation was subjected to off-odour analysis by CG-MS. Five grams of the ground white pepper sample was spiked with 3,4-dimethoxybenzaldehyde (5·1 mg). After extraction with the solution mixture, water: dichloromethane: methanol (4 : 5 : 10, v/v/v), distillation was performed in flash evaporator under vacuum (Jagella and Grosch 1999). This fraction was analysed by gas chromatography-mass spectrometry (Model Hewlett Packard Gas Chromatography (GC) 6890 Mass Spectrometry (MS) 5973), with the following settings: initial temperature – 60°C; final temperature – 243°C; ramping – 3°C per min; run time – 61 min. Front inlet temperature – 220°C; carrier gas – helium; injection volume −1 μl; column used – HD5 cross-linked 5% phenyl methyl siloxane; column length – 30 m; column diameter – 0·32 mm × 0·25 μmol l^{-1} film thickness, constant flow with 4·0 min of solvent delay; mass range 40–400; quadruple temperature – 150°C; mass spectra source temperature – 230°C and interface – 243°C.

Results

Isolation strategies for pericarp-degrading bacteria

In this study, we have isolated 45 bacteria from pepper-associated sources ranging from intact white pepper to decomposed black pepper, on diverse nutrient media (Fig. S1). Most of the isolates obtained were from the medium supplemented with citrus pectin (16 isolates) as the sole carbon source followed by carboxy methyl cellulose (14 isolates) and powdered black pepper pericarp (12 isolates), respectively (Table S1). Among the 45 bacteria evaluated, seven were found to be efficient for decortication of black pepper into white pepper (Fig. 1). These isolates could decorticate more than 60% of black pepper berries into white pepper. Among the type of carbon source used, CMC (two isolates), pectin (two isolates) and pericarp (three isolates)-amended medium yielded more number of potential microbes (seven most efficient bacteria) than other media. Among temperatures, 35°C was found to be more ideal for the microbial conversion as significantly more berries were converted into white pepper. Among the pepper raw materials used, boiled black pepper showed more percentage conversion compared to raw black pepper berries.

Identification of efficient pericarp decorticators

Phenotypic characterization revealed genus identity of seven of the promising bacteria as belonging to *Bacillus, Microbacterium, Acinetobacter* and *Klebsiella* (Table 1). The 16S rDNA sequences obtained from the isolates had nearly 'perfect' match (98–99% similarity) with sequences of their corresponding entries in GenBank, as determined using BLAST (Sequence Match, ver. 2.7). Bacterial isolates IISR WP19 was identified as *Kl. pneumoniae* (GenBank accession: JF907695); IISR-WP25 as *Mic. barkeri* (GenBank accession: JF907694); IISR-WP35 as *Ac. baumanii* (GenBank accession: JF907698); IISR-WP33, 34 and 38 as *B. subtilis* (GenBank accessions: JF907697, JN609214, JF907696); and IISR-WP43 as *B. licheniformis* (GenBank accession: JF907699). Partial sequence data for the 16S rRNA gene have been deposited in the GenBank (NCBI) nucleotide sequence

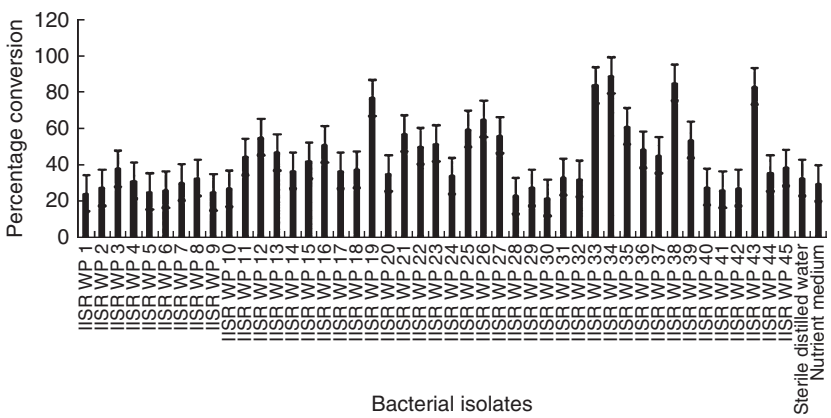

Figure 1 Bacteria-mediated decortication of black pepper into white pepper. Values presented are the mean of three replicates. LSD (P = 0·05) – 3·049.

Table 1 Phenotypic features of bacteria with potential for microbial decortication of pericarp

Isolates	Morphological characters	Biochemical characters	Tentative identity
IISR WP 19	Circular, raised, entire, slimy white colonies, nonmotile, rod, Gram −ve, nonspore formers	Indole (−), methyl red (−), Voges–Proskauer (+), citrate (+), catalase (+), oxidase (−), urease (+), H_2S production (−), pectin hydrolysis (−), CMC hydrolysis (−) xylan hydrolysis (−) casein hydrolysis (−), starch hydrolysis (−), carbon utilization–glucose (+), sucrose (+), lactose (+), mannitol (+), sorbitol (+), maltose (+), trehalose (+), dulcitol (−), ribose (+), arabinose (+), raffinose (+), inositol (+), fructose (+), rhaminose (+) galactose (+), xylose (+), ribitol (+) growth in NaCl-1% (+), 2% (+), 5% (+), 7% (+), 10% (−),growth at 20°C (+), 30°C (+), 40°C (+), 50°C (−), 60°C (−), 70°C (−) 80°C (−).	*Klebsiella* spp.
IISR WP 25	Punctiform, raised, entire, yellow colonies, motile, rod, Gram +ve, nonspore formers	Indole (−), methyl red (+), Voges–Proskauer (−), citrate (−), catalase (+), oxidase (−), urease (−), H_2S production (−),pectin hydrolysis (+),CMC hydrolysis (−) xylan hydrolysis (−) casein hydrolysis (+), starch hydrolysis (−), carbon utilization–glucose (+), sucrose (−), lactose (−), mannitol (+), sorbitol (+), maltose (−), trehalose (+), dulcitol (−), ribose (−), arabinose (−), raffinose (+), inositol (+), fructose (−), rhaminose (+) galactose (+), xylose (−), ribitol (−), growth in NaCl-1% (+), 2% (+), 5% (+), 7% (+), 10% (−),growth at 20°C (+), 30°C (+), 40°C (+), 50°C (+), 60°C (−), 70°C (−) 80°C (−)	*Microbacterium* spp.
IISR WP 35	Circular, convex, entire, white colonies, rod, Gram −ve, nonspore formers	Indole (−), methyl red (−), Voges–Proskauer (−), citrate (+), catalase (+), oxidase (+), urease (+), H_2S production (−), pectin hydrolysis (−), CMC hydrolysis (−) xylan hydrolysis(−) casein hydrolysis (−), starch hydrolysis (−), carbon utilization–glucose (−), sucrose (+), lactose (+), mannitol (−), sorbitol (−), maltose (+), trehalose (−), dulcitol (−), ribose (−), arabinose (−), raffinose (−), inositol (−), fructose (−), rhaminose (−) galactose (+), xylose (−), ribitol (−), growth in NaCl-1% (+), 2% (+), 5% (+), 7% (−), 10% (−),growth at 20°C (+),30°C (+), 40°C (+), 50°C (−), 60°C (−), 70°C (−) 80°C (−)	*Acinetobacter* spp.
IISR WP 33, IISR WP 34, IISR WP 38, IISR WP 43	Irregular, flat, lobate, brown colonies, long rod, motile, spore formers	Indole (−), methyl red (−), Voges–Proskauer (+), citrate (+), catalase (+), oxidase (+), urease (−), H_2S production (−), pectin hydrolysis (+), CMC hydrolysis (+) xylan hydrolysis(+) casein hydrolysis (+), starch hydrolysis (+),carbon utilization–glucose (+), sucrose (−), lactose (−), mannitol (+), sorbitol (+), maltose (−), trehalose (+), dulcitol (−), ribose (−), arabinose (−), raffinose (+), inositol (+), fructose (+), rhaminose (+) galactose (+), xylose (−), ribitol (−),growth in NaCl-1% (+), 2% (+), 5% (+), 7% (+), 10% (+),growth at 20°C (+), 30°C (+), 40°C (+), 50°C (+), 60°C (+), 70°C (+) 80°C (−)	*Bacillus* spp.

database library. Five of the isolates were deposited in Microbial Type Culture Collection at the Institute of Microbial Technology (IMTECH) under CSIR, Chandigarh, India, and assigned MTCC identity numbers MTCC5404 for *Mic. barkeri;* MTCC5405-5407 for *B. subtilis;* and MTCC5408 for *B. licheniformis*. The genera *Acinetobacter* and *Klebsiella* were not deposited.

Large-scale evaluation of efficient decorticators

The efficient decorticators were further evaluated for decortication and the consequent preparation of white pepper from 15 kg of raw fresh berries (*cv. Panniyur 1*). It was observed that the bacteria could soften the pericarp within 5 days of incubation at 35 ± 2°C when

$10^{9–10}$ cells ml^{-1} were used. The pH fluctuation in the fermenting medium was from 6·0 on day 1 to 4·5 on day 5. The fleshy pericarp of the berries was soft enough to be removed mechanically by rubbing them against each other, and the remnants of the pericarp were easily removed by jet of water (Fig. S2). The resultant white pepper kernels were dried in sunlight for 3 days and size sorted using 4·25-mm sieve. The decortication was found to be 98% for IISR WP19, IISR WP35, 99% for IISR WP25 and 100% for IISR WP33, IISR WP34, IISR WP38 and IISR WP43. Significantly high recovery of white pepper was obtained with *Mic. barkeri* (IISR WP 25), *B. subtilis* (IISR WP 33, 34 & 38) and *B. licheniformis* (IISR WP 43) (Table 2).

Assay for hydrolytic enzymes

The hydrolytic enzymes presumed to play a role in decortication are pectinase, cellulase, xylanase, amylase and protease (Table 3; Fig. S3). *Bacillus subtilis* isolates IISR WP 33, 34 and 38 and *B. licheniformis* IISR WP43 produced all the five enzymes assayed. The other bacterium *Mic. barkeri* IISR WP 25 produced only two enzymes viz., pectinase and amylase where as the strains of *Kl. pneumoniae* IISR WP 19 and *Ac. baumanii* IISR WP 35 did not produce any of the enzymes assayed. This observation is in accordance with their decortication efficiency. The *Bacillus* as a genus recorded higher decortication and the consequent white pepper yield than other bacteria.

Physical and Biochemical quality parameters

The creamy white pepper berries obtained from matured fresh pepper (*cv. Panniyur 1*) and subsequently dried under sunlight was found to have superior physical parameters such as colour, texture, odour and appearance (Fig. 2). The moisture content and bulk density of the finished white pepper was 10–12 and 56–57·5%, respectively. The data indicate that the chemical composition of the white pepper, such as oleoresin, piperine, starch and oil, obtained from microbial fermentation by various bacteria are nearly identical (Table 4). Piperine content in microbially converted white pepper was estimated

Table 2 Yield of white pepper obtained from bacteria-mediated decortication of fresh berries of pepper

Bacterial Isolates	Fresh pepper fresh weight (kg)	White pepper dry weight (kg)	*Decortication (%)	†Recovery (%)
IISR WP 19	15·0	2·95	98 (81·9)	19·6 (26·3)
IISR WP 25	15·0	3·79	99 (84·3)	25·3 (30·2)
IISR WP 33	15·0	4·02	100 (90·0)	26·4 (31·0)
IISR WP 34	15·0	3·84	100 (90·0)	25·6 (30·4)
IISR WP 35	15·0	3·42	98 (81·9)	22·8 (28·5)
IISR WP 38	15·0	3·79	100 (90·0)	25·2 (30·1)
IISR WP 43	15·0	4·00	100 (90·0)	26·7 (31·1)
Mock	15·0	1·08	46 (42·7)	7·2 (15·6)
CD at $P = 0·05$		2·560	2·844	2·738

Data in the parenthesis are arc sine-transformed values.
*Decortication (%) – Ratio between white pepper obtained and total berries fermented by count in a parallel experiment using a sample of one hundred berries replicated thrice.
†Recovery (%) – Ratio between white pepper obtained and total berries fermented by weight; Refer Fig. S2 for details.

Table 3 Production of hydrolytic enzymes by efficient decorticators

		Enzyme secretion as indicated by halo around bacterial spot				
Bacterial isolates	Species identity	Pectinase	Cellulase	Xylanase	Amylase	Protease
IISRWP33	*Bacillus subtilis*	+	+	+	+	+
IISRWP34	*B. subtilis*	+	+	+	+	+
IISRWP38	*B. subtilis*	+	+	+	+	+
IISRWP43	*Bacillus licheniformis*	+	+	+	+	+
IISRWP25	*Microbacterium barkeri*	+	–	–	+	–
IISRWP35	*Acinetobacter baumanii*	–	–	–	–	–
IISRWP19	*Klebsiella pneumoniae*	–	–	–	–	–

(+)-Enzyme production (–)-no enzyme production; Refer: Fig. S3.

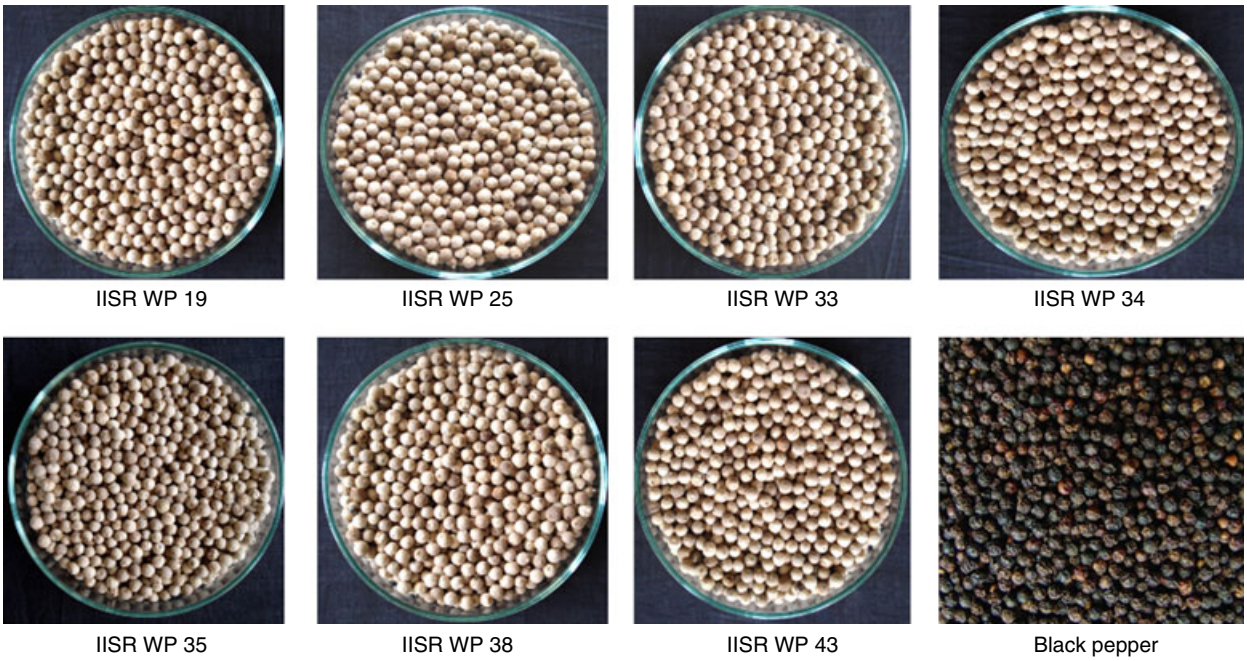

Figure 2 Physical appearance of whiter pepper obtained from bacteria-mediated decortication of fresh berries of pepper.

Table 4 Physical quality parameters and chemical constituents of *white pepper prepared by bacterial fermentation of fresh berries of pepper

	Physical			Chemical			
*Bacteria deployed for decortication	Bulk density (g/1000 ml)	Moisture (%)	Colour (Fig. 2)	Oleoresin (%)	Piperine (%)	Starch (%)	Oil (%)
Klebsiella pneumoniae IISR WP19	568	11·0	+++	8·3	3·6	61·84	2·3
Microbacterium barkeri IISR WP25	570	12·0	+++	8·6	3·4	60·72	2·6
Bacillus subtilis IISR WP33	570	11·8	++++	8·5	3·8	61·50	2·3
B. subtilis IISR WP34	557	10·6	++++	7·9	3·5	58·80	2·3
Acinetobacter baumanii IISR WP35	560	11·9	+++	8·6	3·7	59·90	2·3
B. subtilis IISR WP38	567	12·0	++++	8·0	3·4	55·50	2·3
Bacillus licheniformis IISR WP43	575	12·0	++++	8·2	3·5	58·90	2·6
Black pepper	560	10·2	–	10·5	3·8	43·00	3·3

–, Black; +, brown; ++, dirty white; +++, half white; ++++, bright white.
***White pepper obtained from bacteria-mediated decortication**

using a reverse-phase high-performance liquid chromatographic (HPLC) method. Quantization, based on peak areas, was achieved with reference to purified piperine as standard. With the help of multi-wavelength detector, the spectrum of standard piperine peak was compared with piperine peak which comes from white pepper samples. The piperine content in white pepper was in the range of 3·4–3·7%, while the piperine content in black pepper was 3·8%. The other chemical constituents of white pepper are in the range of 55–60% for starch; 7·9–8·8% for oleoresin, 2·3–2·6% for essential oil (Fig. S4). The results presented in Table 4 indicated that none of the essential quality parameters of white pepper are altered due to microbial fermentation, mediated by bacterial flora belonging to *Bacillus, Microbacterium, Klebsiella* and *Acinetobacter*.

Analysis of volatile oils in white pepper obtained from microbial decortication process:

The volatile components of microbially decorticated white pepper were analysed and compared with that of black pepper. Results of 25 major volatiles identified in the essential oils of white pepper and black pepper

showed dominance of mono- and sesquiterpene hydrocarbons, as presented in Table S2. Steam-distilled pepper oils usually contain about 70–80% monoterpene hydrocarbons, 20–30% sesquiterpene hydrocarbons and less than 4% oxygenated constituents. The important compounds in pepper oils were linalool, δ-3-carene, α-pinene, myrcene, β-caryophyllene, α-phellandrene, limonene, terpinolene, β-pinene and humulene. The GC/MS data of the present investigation showed that the major chemical constituents of essential oil of white pepper produced from the Panniyur 1 cultivar are α-pinene (8·04–8·52%), sabinene (21·1–22·8%), myrcene (2·5–2·6%), limonene (22·1–22·6%) and β-caryophyllene (8·3–9·2%) (Table S2; Fig. S5).

Analysis of off-odour compound – skatole

We have analysed one-year-stored microbially decorticated white pepper for 3-methylindole (skatole), the major compound responsible for off-odour. None of the white pepper preparation from bacteria-mediated decortication in our study showed positive result for skatole. Benzaldehyde, 3, 4-dimethoxy; Benzene, 4-(dimethoxymethyl)-1, 2-dimethoxy-; Benzoic acid, 4-hydroxy-3,5-dimethoxy-, hydrazide were detected in the samples (Table 5; Fig. 3).

Discussion

Black pepper is predominantly cultivated in Vietnam, Indonesia, Brazil, India and Malaysia which collectively contributed 3, 27 000 tons of produce to international trade valued at $130M in the year 2012 (IPC 2012). Contribution of white pepper to the global pepper production is 65 000 MT of white pepper (IPC 2012). White pepper, the choice of Europe, Middle East and USA, is produced from matured fresh pepper by a process termed as 'retting'. This process not only consumes enormous quantum of water but also pollutes it and its environment besides loss of many days, often extends weeks to months to complete the process. The traditional method of white pepper production exploits naturally occurring unknown microbial resources such as bacterial and fungal flora for maceration of the pericarp, which cannot be considered as a standard operating procedure for industrial production of white pepper. The present paper highlights the strategies for isolation of potential bacterial flora for microbial conversion of ripened fresh pepper into white pepper.

Isolation and evaluation of bacteria

In the present study, 45 bacteria isolated from various pepper-associated sources on diverse carbon sources were evaluated for its efficiency to decorticate black pepper pericarp. Among them, three most efficient decorticators were isolated on powdered black pepper pericarp-amended medium. The present study further indicated that a temperature of 35°C and neutral pH was optimum for the fermentation process. Gopinathan and Manilal (2004) reported that temperature ranging from 35 to 40°C was ideal for pectinase action to degrade pepper skin in controlled fermentation conditions. Among the pepper raw materials used for the initial evaluation, boiled black pepper showed more percentage conversion over the unboiled black pepper, indicating a need for softening of tissues for decortication of pepper.

Table 5 GC-MS analysis of white pepper prepared by bacterial fermentation for off-odour compound, Skatole

*White pepper samples	Retention time	Area	Chemical compound detected in the white pepper	Traces of off-odour compound methyl indole (Skatole)
K. pneumoniae IISRWP19	23·660	49·92	Benzaldehyde, 3,4-dimethoxy	Nil
	26·880	50·08	Benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-	
Microbacterium barkeri IISRWP25	23·456	39·80	Benzaldehyde, 3,4-dimethoxy	Nil
	26·761	60·20	Benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-	
Bacillus subtilis IISRWP33	23·661	40·73	Benzaldehyde, 3,4-dimethoxy	Nil
	26·921	59·27	Benzoic acid, 4-hydroxy-3,5-dimeth oxy-, hydrazide	
B. subtilis IISRWP34	23·648	47·26	Benzaldehyde, 3,4-dimethoxy	Nil
	26·875	52·74	Benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-	
Acinetobacter baumanii ISRWP35	23·661	40·73	Benzaldehyde, 3,4-dimethoxy	Nil
	26·921	59·27	Benzoic acid, 4-hydroxy-3,5-dimeth oxy-, hydrazide	
B. subtilis IISRWP38	23·648	47·26	Benzaldehyde, 3,4-dimethoxy	Nil
	26·875	52·74	Benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-	
Bacillus licheniformis IISRWP43	23·661	40·73	Benzaldehyde, 3,4-dimethoxy	Nil
	26·875	52·74	Benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-	

*White pepper obtained from bacteria-mediated decortication.

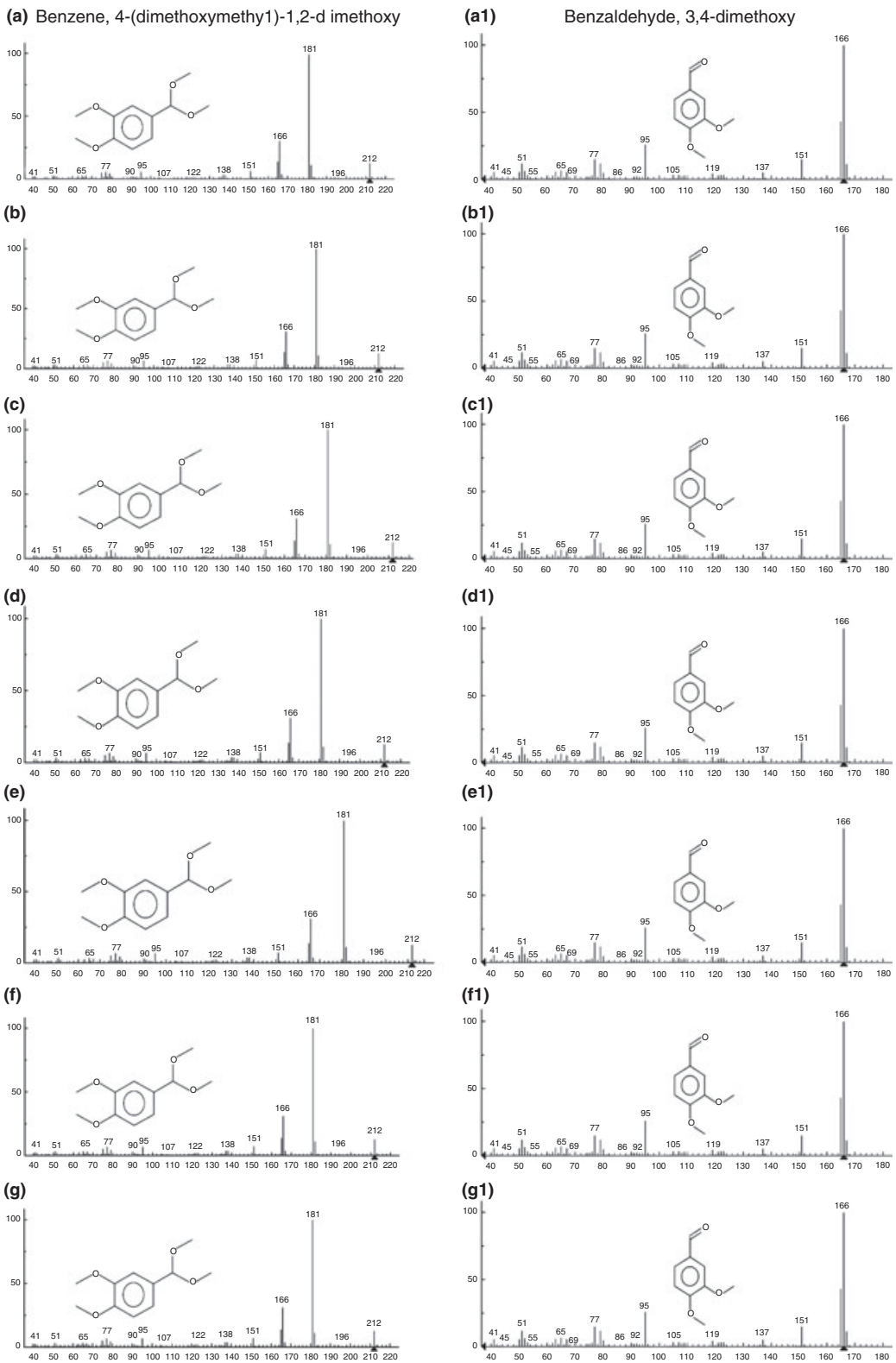

Figure 3 GC-MS chromatograph indicating the absence of skatole in white pepper produced by the selective bacteria-mediated decortication of fresh berries of pepper (a, a1) *Klebsiella pneumoniae* IISRWP19 (b, b1) *Microbacterium barkeri* IISRWP25 (c, c1) *Bacillus subtilis* IISRWP33 (d, d1) *B. subtilis* IISRWP34 (e, e1) *Acinetobacter baumanii* IISRWP35 (f, f1) *B. subtilis* IISRWP38 (g, g1) *Bacillus licheniformis* IISRWP43.

Identification of bacteria

The bacteria were identified by comparing the results of both phenotypic characters and 16S rDNA sequences. Seven shortlisted strains analysed had 'perfect' match (similarity, 98–99%) with ribosomal sequences of *B. subtilis* (IISR WP 33, 34, 38); *B. licheniformis* (IISR WP 43); *Ac. baumanii* (IISR WP 35); *Kl. pneumoniae* (IISR WP 19); and *Mic. barkeri* (IISR WP25). According to Stackebrandt and Goebel (1994), organisms showing less than 97% 16S rDNA sequence similarity will have less than 70% DNA–DNA relatedness, and according to the recommendations for species delineation, these strains should be considered as belonging to different species. Three distinct strains (IISR WP 33, 34, 38) of *B. subtilis* were found to be very efficient in decortication of fresh pepper berries which has been reported as one of the promising bacteria for the production of white pepper (Gopinathan and Manilal 2005). Interestingly, another closely related species *B. licheniformis* (IISR WP 43) was found to perform at par with *B. subtilis* for the production of white pepper. The functional similarity between *B. licheniformis* and *B. subtilis* could be attributed to their close genetic similarity as revealed by 16S rDNA and 16S–23S internal transcribed spacer (ITS) nucleotide sequences (Lapidus *et al.* 2002; Xu and Cote 2003).

Evidences for enzyme-mediated decortication

It is well established that the deskinning of pericarp by microbe is due to secretion of hydrolytic enzymes especially the pectinase. Pectic substances along with other cellulosic material play an important role in structural integrity of plant tissues. Softening makes the tissue suitable for enzymatic action of the bacterium. The present investigation revealed that fresh berries were more amendable for decortication and the consequent white pepper preparation than the dried black pepper berries. The bacterial isolates, particularly *B. subtilis* and *B. licheniformis,* secreted cell wall-degrading enzymes such as pectinase, cellulase, xylanase, amylase and protease that are known to degrade the cell wall component of pericarp. The key enzymes attributed for the decortication process were pectinase (Gopinathan and Manilal 2004) and cellulase (Thankamony *et al.* 1999). During the decortication process, pectin, the intercellular cementing substance, present in the pulpy upper mesocarpic area of pepper skin is degraded and breaks apart from the core. Purified pectinase secreted by *B. licheniformis* IISR WP43 is found to have superior enzymatic properties (Vinod *et al.* 2013).

Quality parameters of white pepper obtained from bacterial decortication of fresh berries

Export-related quality parameters expected of white pepper prepared from various production technologies are its spherical with fairly smooth surface; pale white to brownish white appearance; complete absence of off-odour and low microbial count. The bulk density of white pepper should be 570 g/1000 ml (±20) with less than 2% light berries (ASTA 1968). Our targeted bacterial decortication yielded white pepper that was creamy white colour; spherical smooth surface with bulk density ranging from 557 g to 575 g/1000 ml. The moisture content of the white pepper was 12–13%. ASTA recommends moisture levels of 12% for black pepper and 14% for white pepper for international trade.

White pepper contains about 2·5% essential oil, whose aroma is dominated (max. 80%) by monoterpene hydrocarbons: sabinene, β-pinene, limonene, terpinene, α-pinene, myrcene, Δ3-carene and monoterpene derivatives (borneol, carvone, carvacrol, 1, 8-cineol, linalool). Nijssen *et al.* (1996) showed over 250 volatile compounds in essential oils of black pepper. Volatile oil composition in pepper showed significant variation among different cultivars (Lewis *et al.* 1969a,b; Datta *et al.* 1971; Richard *et al.* 1971; Russell and Else 1973). We observed marginal reduction in essential oil in white pepper prepared from bacterial decortication as compared to raw black pepper (3·3%) that could be due to loss of volatile oil-bearing pericarp-associated cells which were removed during the process of decortication. Decrease in volatile oil content in the white pepper as compared to black pepper during decortication process has been reported earlier (Govindarajan 1977; Verghese 1989), which was attributed to the loss of pericarp-associated volatile oil-bearing cells (Mangalakumari *et al.* 1984; Gopalam *et al.* 1991).

Lewis *et al.* (1969a) found that monoterpene present in the oil is responsible for contributing strong peppery odour, whereas caryophyllene is responsible for sweet floral odour in pepper. Buckle *et al.* (1985) reported that the levels of α-phellandrene and limonene in black pepper oil were higher than those in white pepper oil, which could be the reason for milder flavour of white pepper than that of the black pepper. GC-MS analysis of white pepper volatile oil by Sunita *et al.* (2013) reported that β-caryophyllene (16·0%), sabinene (12·6%), limonene (11·9%) and torreyol (9·3%) were the major components with many minor components. Taken together, it could be concluded that the fermenting organism did not influence the important trade-linked quality parameters, secondary metabolites and aroma-contributing essential oil constituents such as oleoresin and piperine.

Off-odour components in white pepper: A 'consumer acceptance'-related issue needs technological intervention

One of the major issues of white pepper trade is its offensive faecal odour and the consequent consumer rejection. Jagella and Grosch (1999) suggest that the faecal off-odour is the main quality issue of white pepper at international trade. The off-odour accumulation on pepper is due to prolonged and extended soaking in natural stream of water which facilitates colonization by unknown microbes including toxic coliform bacteria. Therefore, short duration of microbial fermentation would be advantageous for the production of high-quality white pepper. Furthermore, the deployment of a known microbial species as targeted decortication would be highly advantageous for ensuring high-quality white pepper. Our data on GC-MS analysis of white pepper obtained from bacterial fermentation did not show the presence of the off-odour compound, skatole. Whitfield *et al.* (1982) have reported that bacterial degradation of the amino acids tyrosine and tryptophan contributes for off-odour compounds like *p*-cresol and skatole in potatoes. Bacteria preferentially attack easily utilizable carbohydrates over recalcitrant lignins, thus increasing the relative amount of 2-methoxy phenol. Accumulation of 1, 2-dimethoxy benzene may result from lignin that was relatively refractory towards bacterial degradation. These compounds are not genuine pepper-based compounds; the fermentation is the precursor for this kind of off-odour compound formation (Steinhaus and Schieberle 2005). Our study clearly revealed the benefit of deploying single bacterial species for decortication of pepper that has led to the production of high-quality white pepper free from off-odour.

Bacillus-mediated decortication technology for white pepper production – a clean technology for developing world

In the present investigation, a total of four different strains of *Bacillus* were found to be very efficient in decortication of fresh pepper, which has been reported as one of the promising bacteria for the production of white pepper (Gopinathan and Manilal 2005). *Bacillus licheniformis* was found to perform marginally better than that of *B. subtilis* for the production of white pepper from black or fresh pepper that is known to produce extracellular enzymes mainly pectinase, cellulase and xylanase in large quantity. Superior performance of *Bacillus* for decortication of black or fresh berries could be attributed to secretion of diverse hydrolytic enzymes. *Bacillus licheniformis* is one of the most important industrial organisms used in the biotechnology industry to manufacture several enzymes, antibiotics, biochemicals and consumer products (Rey *et al.* 2004).

The specific bacterial species-based fermentation method for the preparation of white pepper yielded superior-quality white pepper at a relatively shorter duration of 5 days as compared to other methods reported or patented elsewhere. The short duration of bacterial action for decortication would significantly reduce water consumption and operational cost for the production of white pepper. The physical quality parameters such as colour, texture, bulk density and appearance were in the acceptable level. The volatile oil and piperine contents which are responsible for the aroma were conspicuously enhanced in the produce. Significantly, off-odour compound, skatole, could not be detected in the one-year-stored white pepper indicating the potential of the process for further scale-up. The bacterial action on the berries showed no significant influence on secondary metabolites such as essential oil constituents, oleoresin and piperine content. Utilization of *Bacillus* species especially *B. licheniformis* MTCC 5408 for the production of white pepper from ripened fresh or black pepper by fermentation is one of the most significant findings of the work, and the same is patent filed with Indian Patent Office for patent protection (Kumar *et al.* 2012). Perusal of records reveals that this is the first record of *B. licheniformis* for the production of white pepper from black or fresh pepper by decortication of pericarp.

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Conflict of interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The colony morphology of the isolated strains on 2, 3, 5-tri phenyl tetrazolium chloride (TTC)-amended nutrient agar medium.

Figure S2 Production technology for white pepper from matured green pepper by bacterial fermentation.

Figure S3 Plate assay for the extracellular enzyme production by the strains.

Figure S4 HPLC profiling of piperine content of white pepper produced by microbial fermentation. (a) Black pepper control, (b) *Klebsiella pneumonia* IISRWP19, (c) *Microbacterium barkeri* IISRWP25, (d) *Bacillus subtilis* IISRWP33, (e) *B. subtilis* IISRWP34, (f) *Acinetobacter baumanii* IISRWP35, (g) *B. subtilis* IISRWP38, (h) *Bacillus licheniformis* IISRWP43.

Figure S5 GC-MS chromatograph showing the essential oil composition of white pepper produced by the selective bacterial fermentation.

Table S1 Source of isolation, media used and isolates obtained.

Table S2 Analysis of essential oil in white pepper obtained from bacteria-mediated decortication of pepper berries.