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Molecular Identification of a bacterial pathogen reducing shelf life of *Agaricus bisporus* and its prevention

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Agaricus bisporus(common name "button mushroom") is an edible basidiomycetous mushroom, widely consumed in different parts of the world. Microbial spoilage of *Agaricus bisporus* causes "Bacterial blotch" [Figure 1] and surface slime formation[Figure 2] on the pileus. In our study, we characterized the bacteria that caused microbial spoilage in a population of *Agaricus bisporus* collected from a local market in Kolkata, West Bengal. Initial characterization was performed using Gram staining, followed by biochemical tests (catalase, oxidase, nitrate reductase, IMViC, TSI reduction, starch hydrolysis, gelatin hydrolysis, carbohydrate fermentation) and motility tests,following culture of the exudate from spoilt mushrooms. Final characterization was achieved by performing 16S rDNA analysis. The spoilage organism was found to be a bacterial pathogen, *Stenotrophomonus maltophilia strain 1.22*, based on nucleotide homology and phylogenetic analysis. The second component of our study focussed on increasing the shelf life of another population of fresh *Agaricus bisporus* collected from the same source, exposed to the same bacterial pathogen. By employing the processes of acid blanching (acetic acid) and antibiotic spraying (Streptomycin), the shelf life of fresh button mushrooms, at chilled storage conditions(4°C)was increased appreciably, by 3 to 16 days. Increasing the shelf life of button mushroom has commercial importance as *Agaricus bisporus* is very susceptible to spoilage by enzymatic or microbial methods.

Key words : *Agaricus bisporus,* Microbial spoilage, Bacterial blotch, *Stenotrophomonas maltophilia strain 1.22,* Shelf life

INTRODUCTION

Agaricus bisporus has a short shelf life because of the rapid respiration rate. Mushroom spoilage occurs by dehydration, microbial, and enzymatic processes. Bacterial growth on *Agaricus bisporus* (Singh *et al.,* 2007) is conducive to enzymatic browning, mainly initiated by tyrosinase, resulting in the formation of brown pigments on the pileus. Studies on characterisation of bacteria causing microbial spoilage of *Agaricus bisporus* mainly focus on *Pseudomonas* sp. as experiments have reported that *Pseudomonas tolaasii* (Tsuneda *et al.,* 2010) is capable of producing a toxin that lyses mushrooms, resulting in bacterial blotch (brown pigmentation, lesions) on the pileus. Prevention of mushroom spoilage is done by the chilled storage of mushrooms. In this investigation, physical, chemical and molecular methods have been used, to identify the causative agent of microbial spoilage of a population of *Agaricus bisporus.* The spoilage organism follows Koch's postulates and is a potential pathogen (Schneider *et al.*, 2010). Molecular identification techniques (16S rDNA analysis) have conclusively identified the pathogen. Enhancement of pretreatment methods (Lin *et al,* 2001) that are presently used is necessary, as restricting bacterial growth is not sufficient to prevent spoilage. The designing of a novel approach for increasing shelf life of *Agaricus bisporus* was required. We used the techniques of acid blanching (acetic acid) (Coskuner *et al.,* 2000) and antibiotic spraying (Streptomycin), and both in conjunction, to ascertain the best method of preventing button mushroom spoilage (Singh *et al.*, 2007). Pretreatment methods have been corroborated with macroscopic observation.

MATERIALS AND METHODS

(i) Preliminary Characterization of spoilage organism included microscopic study (Gram

staining), and Hanging drop test for microbial motility. Koch's postulates were established by infection of fresh *Agaricus bisporus*. Source of *A. bisporus:* local market in Manali valley, Himachal Pradesh.

Biochemical characterization included Catalase, Oxidase, Nitrate reduction, IMVIC, Urease, TSI reduction, Carbohydrate fermentation, Gelatin liquefaction, and Starch Hydrolysis tests.

Methodology of isolation

Specific "bacterial blotch" symptoms of spoilt *Agaricus bisporus* were identified. The exudate from the spoilt mushrooms was inoculated into Nutrient Agar plates and incubated at 37°C for 24-48 hrs. The bacterial colonies obtained were repeatedly subcultured in more NA plates until a pure or axenic culture is obtained.

Maintenance of pure culture

Bacteria that were isolated from the pure culture (NA slants) were maintained by the process of repeated subculturing. Subculturing of the pure culture was done using the streak plate method.

Microscopic study

Gram staining of the cultured bacteria was done using standard techniques.

Hanging drop test for microbial motility

The test for microbial motility was performed using standard techniques.

Establishment of Koch's postulates

Pathogenecity of the isolated bacterial strain has been proved by the Koch's postulates. Fresh *Agaricus bisporus* that were suspended in the experimental bacterial suspension for 48-72 hrs in a sterilized environment were seen to be infected, showing similar symptoms of bacterial blotch and surface slime.

Biochemical Characterization

Catalase test

A drop of 3% H_2O_2 is taken on a clean glass slide and with the help of sterilized glass rod; growth from an isolated colony was transferred onto the drop of hydrogen peroxide. Appearance of effervescence constitutes a positive test.

Oxidase test

A strip of filter paper was soaked with 1% of oxidase reagent (N,N,N,N-Tetramethyl para phenylene diamine dihydrochloride) and then at once used by rubbing a speck of culture on its surface with a glass rod. A positive reaction is indicated by the appearance of an intense deep blue colour in 5-10 seconds.

Nitrate reduction test

Autoclaved nitrate broth was inoculated with the experimental organism and kept in the BOD incubator at 37°C for 24 hrs. Then sulfanilic acid and alpha-naphthylamine is added for generation of red colour. If no red colour appears, then a pinch of zinc dust is added.

Indole production test

Autoclaved tryptophan broth was inoculated with the test organism and incubated for 24-48 hrs at 37°C. After the incubation period, 0.5 ml of Kovac's reagent (para dimethyl amino benzaldehyde in amyl alcohol) was added with dropper from the walls of the tube without shaking. The development of a reddish coloured ring in the upper alcohol layer of the broth was taken as a positive result.

Methyl red test

Autoclaved dextrose phosphate broth is inoculated with the experimental organism and kept in the BOD incubator for 24 hrs and to it, methyl red indicator was added to the culture without shaking for the development of red colour.

Voges Proskauer test

Autoclaved dextrose phosphate broth is inoculated with the experimental organism and kept in the BOD incubator for 24 hrs and to it, alpha-napththol was added, followed by 40% KOH solution, and shaken continuously for 15 minutes. Development of red colour indicates positive reaction, and development of copper colour indicates negative reaction.

Citrate Utilization test

Simmon's citrate slant was prepared and the ex-

perimental organism was streaked in the slant, incubated for 24-48 hrs and checked for the development of deep blue colouration.

Urease test

Christensen's urease slant was prepared, and the experimental organisms were streaked on the slant, incubated for 18-24 hrs at 37°C in the BOD incubator for the development of pink colour.

TSI reduction test

TSI slants are prepared and inoculated with the experimental organism both in the slant and stab, and after incubation, they are observed for colour changes.

Carbohydrate fermentation test

Autoclaved fermentation media (specific carbohydrates- glucose, sucrose, lactose, and mannitol) is taken into fermentation tubes, inoculated with the experimental organism, and incubated in the BOD incubator at 37°C for 24-48 hrs. The study of acid production (colour change to yellow) and gas production (formation of gas bubbles) is done and compared with the control.

Gelatin liquefaction test

Autoclaved gelatin hydrolysis medium was inoculated with the experimental organism and incubated for 24-48 hrs at 37°C in the BOD incubator, and then kept in ice for 15-20 minutes. Liquefaction of the medium indicates hydrolysis of gelatin.

Starch Hydrolysis

Autoclaved starch hydrolysis media is inoculated with the experimental organism and incubated for 24 hrs at 37°C in the BOD incubator. This is followed by flooding the Petri plate with a thin layer of Iodine. If a clear zone is detected around the colony, it indicates starch hydrolysis.

16S rDNA analysis of the spoilage organism

DNA was isolated from pure culture. Its quality was evaluated on 1.2% Agarose Gel, single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR from isolated DNA. Single discrete PCR amplicon band of 1500 bp was observed when resolved. PCR amplicon was purified. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1428 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. 16S rDNA gene sequence was used to perform BLAST with the nrdatabase of NCBI Gen Bank database. Based on maximum identity score first ten sequencaes were selected and aligned using multiple alignment software program. Clustal W. Distance matrix was generated using RDP database and phylogenetic tree was constructed using MEGA 4.

(iii) Increasing shelf life of Agaricus bisporus

Fresh *Agaricus bisporus* was taken from the same source, and to it 0.001-0.002 ml of bacterial suspension was introduced with the help of calibrated loop and then it was treated with varying concentrations of acetic acid (acid blanching) and antibiotic Streptomycin (antibiotic spraying). Methods of acid blanching and antibiotic spraying have also been used in combination. All setups are wrapped in standard perforated viskase film and maintained at chilled storage conditions (4°C), along with control. Dry weight, fresh weight, and macroscopic observation of *Agaricus bisporus* is noted for 3 weeks (at 24 hr intervals). Spoilage is denoted by wilting of the mushrooms, surface slime, exudate production, bacterial contamination and bacterial blotch formation on the pileus (Srivastava *et al.,* 2010). Effects of the pretreatments were observed, observations were recorded. Shelf life of each setup was compared to the control, to ascertain the increase in shelf life of the setups, and the efficacy of pretreatment methods.

RESULTS

Preliminary characterization of spoilage organism

Population of fresh *Agaricus bisporus* is found to be spoilt by bacteria causing bacterial blotch, browning and surface slime formation. Initial characterization reveals the organism to be thin, motile, Gram negative rods (Table 1) that grow well in both liquid and solid medium, Nutrient Broth and Nutrient Agar.

Table 1: Morphological and culture characteristics of the bacteria

Characteristic	Isolated Strain
Shape	Thin rods
Length in microns	1.11 µ
Gram nature	Gram negative
Motility	Motile
Culture Characteristics	
Nutrient Broth	Surface growth present, clouding less
Nutrient Agar	Colonies are off-white, round, entire, raised and opaque

Table 2 : Biochemical characteristics of the bacteria

Characteristic	Isolated Strain
Catalase test	Positive
Oxidase test	Negative
Nitrate reduction test	Positive
Indole production test	Negative
Methyl red test	Negative
Voges Proskauer test	Negative
Citrate Utilization test	Positive
Urease test	Negative
TSI reduction test	Negative
Carbohydrate fermentation test	▪Negative
▪Glucose	▪Negative
▪Sucrose	▪Negative
▪Lactose	▪Negative
▪Mannitol	
Gelatin liquefaction test	Positive
Starch Hydrolysis	Negative

Biochemical characterization of spoilage organism

An unusual characteristic of the organism is that it is catalase-positive and oxidase-negative. Positive nitrate reduction test and positive citrate utilization test is exhibited. The organism is unable to ferment carbohydrates, yet it can liquefy gelatin (Table 2). This demonstrates the versatility of the spoilage organism.

Morphological and biochemical tests are insufficient to identify the spoilage organism. Molecular identification using 16S rDNA has been done as a confirmatory test.

Molecular identification using 16S rDNA analysis

The spoilage organism was found to be a common bacterial pathogen *Stenotrophomonas maltophilia strain 1.22* (GenBank Accession Number: EF426435.1) based on nucleotide homology and phylogenetic analysis (Table 4). *Stenotrophomonas maltophilia* (Su *et al.,* 2010) is ubiquitous in aqueous environments, soil and plants. Information about other close homologs for the microbe can be found from the Alignment View table (Table 3). The % similarity of the present strain with that of closely related homologs has been found to be nearly 99%.

Pretreatments for increasing shelf life

Acid blanching using different concentrations of acetic acid and antibiotic spraying using different concentrations of Streptomycin were investigated. Results reveal that both processes significantly affect the shelf life of button mushrooms. Using these processes, shelf life could be extended by 3 to 16 days (Table 5). Maximum increase in shelf life was seen in case of a sample of *Agaricus bisporus* treated with a combination of 12% acetic acid and 10 mg/ml Streptomycin. This setup showed extended shelf life of 16 days, as compared to the control.

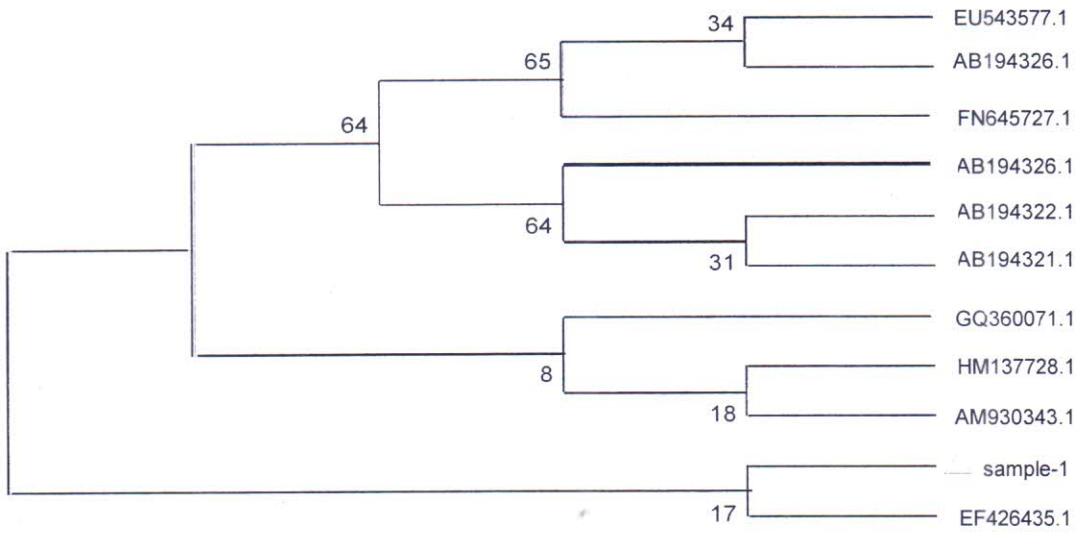
DISCUSSION

The major problem of popularizing *Agaricus bisporus* in this part of the world is high temperature and humidity which results in the infection of button mushroom by common tropical bacteria (Srivastava *et al,* 2010). This is the principal reason behind opting for pretreatment methods for increasing the shelf life of *Agaricus bisporus.* In this investigation, the objective was to identify and characterize the spoilage organism. We carried out culture characteristic of the isolated bacteria. From Table 1 we found that the colonies are off-white, round, entire, raised and opaque. We carried out the morphological characterization. From Table 1 we found that the bacteria is gram-negative, rod and it is motile.We established that the bacteria is pathogenic to *Agaricus bisporus* by establishing the Koch's postulate. From Table 2 we found out the biochemical characteristic of the bacteria.

Table 3: Sequence Producing Significant Alignments on the basis of BLAST data

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HM137728.1	*Stenotrophomonas maltophilia* strain WS8	2638	2638	100%	0.0	100%
GQ360071.1	*Stenotrophomonas maltophilia* strain pp5c	2623	2623	100%	0.0	99%
FN645727.1	*Stenotrophomonas maltophilia*	2617	2617	100%	0.0	99%
AM930343.1	Uncultured bacterium, clone SMQ138	2617	2617	100%	0.0	99%
EU543577.1	*Stenotrophomonas maltophilia*	2617	2617	100%	0.0	99%
EF426435.1	*Stenotrophomonas maltophilia* strain 1.22	2617	2617	100%	0.0	99%
AB194326.1	*Stenotrophomonas maltophilia*, strain:BL-16	2617	2617	100%	0.0	99%
AB194325.1	*Stenotrophomonas maltophilia*, strain:BL-15	2617	2617	100%	0.0	99%
AB194322.1	*Stenotrophomonas maltophilia*, strain:BL-10	2617	2617	100%	0.0	99%
AB194321.1	*Stenotrophomonas maltophilia*, strain:BL-9	2617	2617	100%	0.0	99%

Table 4: Phylogenetic Tree (Evolutionary relationships of 11 taxa)



The molecular identification of the spoilage organism was carried out by 16s r-DNA analysis. From Table 3 and Table 4 we see that the spoilage organism is *Stenotrophomonas maltophilia* strain 1.22.

We tried to increase the shelf-life of the mushroom by treating it with different concentration of acetic acid(acid blanching) and different concentrations of antibiotic Streptomycin(acid spraying). We also used a combination of acid spraying and acid blanching. The experiment was performed at chilling storage temperature of 4°C. From Table 5 we see that as we increase the concentration of acetic acid and antibiotic streptomycin blotching decreases. We also see that there is reduction in the bacterial contamination in extrudate. We found that blotching and bacterial contamination in extrudate is minimum when we use combination of antibiotic and acid.

From Table 5, we see that the shelf life of the mushroom which is treated with combination of both acids and antibiotic is maximum. The shelf-life of mushroom has increased to 16 days when treated with a combination of both acid and antibiotic from 4 days when it was neither treated with any of them. So a combination of both these methods along with a cold storage is best for preserving the mushroom for future consumption.

Table 5: Effect of Streptomycin and acetic acid an the shelf life of *Agaricus bisporus* Chilling Storage Temperature: 4°C

Treatment	Fresh Weight		Colour		Blotching after Spoilage	Exudate Production/ Bacteria In Exudate	Shelf Life (Days)
	Initial Weight (Gms)	Final Weight (Gms)	Initial colour	Final colour			
1.Control	150.12	150.09	White	Dark Brown	Yes	Yes/Yes	
2.Acetic Acid Treatment (Blanching)							
(i) 4%	149.32	149.30	White	Light Brown	Yes	Yes, Profuse/No	3
(ii) 8%	150.20	150.24	White	Yellowish White	No	Yes, Profuse/No	3
(iii) 12%	202.01	200.95	White	Dull White	No	Yes/No	4
3.Antibiotic Treatment With Streptomycin							
(i) 1 ug/ml	49.30	49.32	White	Dull White	No	No/No	3
(ii) 2 ug/ml	66.21	66.03	White	White	No	No/No	6
(iii) 5 ug/ml	53.42	52.65	White	White	No	No/No	7
(iv)10ug/ml	49.82	49.56	White	White	No	No/No	15
4.Antibiotic + Acetic Acid							
(i) 10ug/ml + 8%	111.72	111.42	White	Dull White	No	Yes/No	9
(ii) 10ug/ml + 12%	73.45	73.55	White	White	No	No/No	16

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