SPENT SUBSTRATE FROM MUSHROOM INDUSTRY, A POTENTAIL DYE DECOLOURIZING AGENT

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ABSTRACT

Dye decolourization using spent mushroom substrate (SMS) is influenced by the category of SMS, type of dye, initial dye concentration, form of mushroom mycelia, pH, temperature and the growing media. Among SMS of different mushrooms, the SMS from *P. sajor-caju* exhibited highest population along with variability of both fungi and bacteria. Five fungi viz., *Aspergillus fumigatus*, *Paecilomyces variotii*, *Pichia guilliermondii*, *Schizophyllum commune* and *Pezizomycotina* sp. with potential dye decolourization potential have been recorded to thrive on SMS of different mushrooms by using 5.8S rRNA gene sequencing and BLASTn techniques. Out of these *Schizophyllum commune* from *P. sajor-caju* SMS has been recorded to exhibit highest decolourization potential (95%) with in 10 days against Chicago sky blue. This fungus also exhibited dye decolourization potential of 100, 92.50, 81.60, 73.40 and 67.80% against other dyes like Starch Azure, Reactive blue, Rhodamine B, Orange II sodium salt and Methyl blue, respectively. Similarly, by using 16S rRNA gene sequencing and BLASTn, six potential bacteria viz., *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *Pseudomonas fluorescens*, *Sphingobacterium multivorum* and *Rummelibacillus stabekissi* have also been identified, out of which *B. licheniformis* isolated from *P. sajor-caju* SMS, exhibited highest decolourization potential of 66.10% against Orange II sodium salt, followed by *Bacillus pumilus* (57.7%). Temperature and pH optima of 25ºC and 6.0 respectively, have been recorded for achieving highest level of decolourization through *B. licheniformis*. The study highlights the utility of SMS for unconventional activities like dye decolourization, which indirectly supports its use in bioremediation activities.

Key words: Fungi; Bacteria; Dye decolourization; 16S rRNA; 5.8S rRNA

INTRODUCTION

Synthetic dyes are used in a wide range of industries (textile, pharmaceutical, cosmetic and food industries) [1] and during processing, up to 40% of used dyestuffs are released in to processed water [2], producing a highly colored wastewater that affects aesthetics, transparency and gas solubility in water bodies [3]. Other main factors, which make it a problematic industrial waste, are the mutagenic, carcinogenic and toxic effect of some of these dyes [4], high volume and effluent composition [3], and their resistance towards the conventional methods of wastewater treatment [5]. Currently several physico-chemical methods are in use for decolorization of these wastewaters, but these have limitations of high cost, high salt content in the effluent and difficulties in treating concentrated waste [6]. Being eco-friendly and cost-competitive [7], white rot fungi have been recognized as most efficient, degrading dyes with the involvement of their extracellular lignin-modifying enzymes [8].

Spent Mushroom Substrate (SMS) released after mushroom cultivation, contains residual mushroom biomass along with a rich population of heterotrophic fungi and bacteria, which can act as an inexpensive source of phenoloxidases. SMS also has ability to chemically adsorb organic and inorganic pollutants, while diverse category of microbes it harbors, have capability

of biologically breaking down the organic xenobiotic compounds present in soil and water [9]. In several previous studies, role of extracellular ligninolytic enzymes and microbes from SMS of different mushrooms [10], and mushroom mycelia, especially of *Pleurotus florida*, *P. ostreatus*, *P. flabellatus* and *P. sajor-caju* have been evaluated for their use in dye decolorization activities [3, 11]. This study presents the role of different fungi and bacteria inhabiting the spent substrate of different mushrooms, and temperature and pH of medium in decolurization of different dyes.

MATERIALS AND METHODS

Isolation and identification of spent substrate microflora. Spent substrate from button (*Agaricus bisporus*), oyster (*Pleurotus sajor-caju*) and paddy straw mushroom (*Volvariella volvacea*) collected from Directorate of Mushroom Research, Solan (HP), India was used for isolation of fungi and bacteria. Fungi and bacteria were isolated using the serial dilution and plating method on Potato Dextrose Agar (potato dextrose - 24 g, agar agar - 20 g, distilled water - 1000 ml, pH - 7.2) and Nutrient Agar (beef extract - 3 g, peptone - 5 g, dextrose - 3 g, NaCl - 5 g, agar agar - 15 g, water - 1000 ml, pH - 7.2) medium, respectively and incubating at 30±2ºC for 5 days. Well isolated colonies were further purified by growing on respective media and stored thereafter at 4ºC for further use, after growing them on respective media slants. Purified fungi and bacteria were identified using 5.8S rRNA and 16S rRNA gene sequencing, followed by Nucleotide Basic Local Alignment Search Tool **(**BLASTn) techniques.

Fungal DNA extraction and 5.8S rRNA gene amplification. All fungal cultures were grown separately on Malt Extract Agar petridishes at 32 ± 2°C for 7 days. The mycelia from respective fungus plates was scrapped and placed at -85°C at least for 2 hours, followed by freeze drying for 16-18 hours. The genomic DNA was extracted from approximately 100 mg of freeze dried fungal mycelia by crushing in 1.5 ml micro-centrifuge tubes using micro-pestles. QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH, D-40724 Hilden) was used for DNA extraction as per the protocol supplied by the manufacturer.

The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al* [12] were used to amplify the ITS region of 5.8S ribosomal DNA. PCR amplification was performed in a reaction mixture of 50 µl, containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2.0 mM each), 1 µl each of ITS-1 and ITS-4 primers (0.01 mM), 1 µl glycerol (5%), 2 µl $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). PCR reaction was performed in PCR Master Cycler Gradient in 36 cycles each of 95°C for 1 min, 50°C for 30 sec, 72°C for 1 min 20 sec and final elongation at 72°C for 10 min with lid heating option at 104°C. The presence and yield of PCR amplified amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 1.30 h in 0.5X TBE buffer. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System (Gene Genius, Syngene).

Bacterial DNA extraction, quantification and 16S rRNA gene amplification. All bacterial isolates were grown separately in 10 ml nutrient broth in 20 ml screw caped tubes at 30±2ºC for 2 days. For extraction of total genomic DNA, 1.5 ml log phase broth culture was used and DNA was extracted using BACTOZOL ™ Bacterial DNA Isolation Kit from Molecular Research Centre, Inc., Cincinnati, OH. Extracted DNA samples from different bacterial isolates were run on 1.2% agarose gel (w/v) in 1.0% TBE buffer at 90 V for 1 hr along with quantification marker in one lane. Gel was visualized using Bio Imaging System (Gene Genius, Syngene).

Alternatively, the DNA concentration and purity were also checked by measuring the extinction at A_{260}/A_{280} on UV Visible spectrophotometer. The concentration was calculated assuming that one A_{260} unit is equal to 50 g of double stranded DNA/ml.

Amplicon of 16S rDNA was obtained for each bacterial isolate by PCR amplification employing forward primer (5'-GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and reverse primer (5'-CCG TCA ATT CMT TTG AGT TT-3'). PCR reactions were performed in a reaction mixture of 50 µl containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2 mM each), 1 µl each of Forward primer and Reverse primer (0.01 mM), 1 µl glycerol (5%), 2 µl of $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). The reactions were performed in a PCR Master Cycler Gradient with initial denaturation at 95°C for 3 min, followed by 35 cycles each of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min and final elongation at 72°C for 10 min with lid heating option at 104°C. The presence and yield of specific PCR product (16S rRNA gene) was visualized as per the protocol adopted for 5.8S rRNA gene.

PCR products cleaning up, sequencing, annotation and blasting. PCR amplified amplicons of 5.8S rDNA and 16S rDNA were cleaned up by using RCB kit (Banqiao City, Taipei County 220, Taiwan) for removing of, if any undesired DNA fragments. The cleaned up PCR products were got sequenced using 3730Xl (96 capillary) electrophoresis instruments from Bioserve Biotechnologies, Hyderabad, India.

The received sequences in ab1/SCF format were cleaned up to remove the misleading sequences and were improved upon using Chromas software. The improved consensus sequences were blasted using BLASTn of NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and the species against which highest total score was exhibited, was considered as the species identified.

Different dyes used and decolorization potential of different fungi. Seven dyes (Table 1) were procured from Sigma-Aldrich. Seven fungi belonging to 5 different genera [*Aspergillus fumigatus* (Ab), *Aspergillus fumigatus* (Ps), *Schizophyllum commune* (Ps), *Pezizomycotina* sp. (Ps), *Aspergillus fumigatus* (Vv), *Paecilomyces variotii* (Vv) and *Pichia guilliermondii* (Vv)] and isolated from three different spent substrates (given in parenthesis) were screened for their dye decolourization potential against Chicago sky blue. The dye decolourization potential was studied against 100 ppm concentration of the dye in Potato Dextrose Broth (PDB - potato dextrose, 24 g; distilled water, 1000 ml; pH, 7.2). Five day old culture of different fungi was inoculated separately in dye mixed 100 ml PDB in 250 ml Erlnmeyer flasks and incubated at 25 ± 1°C for 12 days in BOD incubator. Three replications were kept for each treatment keeping un-inoculated flasks as the control treatment. The decolourization if any, was measured as per the protocol elaborated under heading ‘measurement of decolorization extent’.

The potential fungus (*Schizophyllum commune*) isolated from *P. sajor-caju* spent substrate was further studied for its dye decolourization potential against 6 different dyes (Methyl blue, Orange II Sodium salt, Rhodamine B, Azure B, Reactive blue and Starch Azure). The decolourization potential was evaluated against 100 ppm concentration of each dye in 100 ml PDB filled 250 ml Erlnmeyer flasks. Five day old culture of the test fungus was inoculated separately in each flask and the flasks were incubated at 25 ± 1°C for 18 days in BOD incubator. Three flasks were kept for each treatment by keeping flasks devoid of fungal inoculation as the control treatment. The decolourization was measured as per the protocol elaborated under heading ‘measurement of decolorization extent’.

Table 1: Details of different dyes used in study

Dye	Chemical formula	CAS No.	λ max (nm)	Reference
Rhodamine B	$C_{28}H_{31}N_2O_3Cl$	81-88-9	543	[13]
Methyl Blue	$C_{16}H_{18}ClN_3S$	61-73-4	655	[14]
Orange II sodium salt	$C_{16}H_{11}N_2NaO_4S$	633-96-5	550	[15]
Azure B	$C_{15}H_{16}N_3S^+$	531-55-5	648	[16]
Reactive Blue	$C_{22}H_{16}N_2Na_2O_{11}S_3$	2580-78-1	550	[17]
Starch Azure	$C_{36}H_{55}NO_{28}$	66068-38-0	650	[18]
Chicago Sky Blue 6B	$C_{34}H_{24}N_6Na_4O_{16}S_4$	2610-05-1	650	[19]

Dye decolorization potential of different bacteria. Eight bacterial isolates belonging to 5 different genera [*Sphingobacterium multivorum*-1 (Ab), *Sphingobacterium multivorum*-2 (Ab), *Bacillus pumilus* (Ab), *Rummelibacillus stabekisii* (Ps), *B. licheniformis* (Ps), *B. subtilis* (Ps), *B. pumilus*-1 (Vv) and *B. pumilus*-2 (Vv)] and isolated from three different spent substrates (given in parenthesis) were screened for their dye decolourization potential against 100 ppm concentration of Orange II Sodium salt in PDB. Two day old culture ($\times 10^{-6}$/ml) of different bacteria was inoculated @ 0.1 ml separately in dye mixed 100 ml PDB filled in 250 ml Erlnmeyer flasks and the flasks were incubated at 25 ± 1°C for 8 days in BOD incubator. Three replications were kept for each treatment by keeping un-inoculated flasks as the control treatment. Decolourization was recorded like in case of earlier steps.

The potential bacteria (*B. licheniformis* and *B. subtilis*) isolated from *P. sajor-caju* spent substrate were evaluated for their dye decolourization potential against 2 additional dyes (Azure B and Methyl blue). The decolourization potential was studied against 100 ppm concentration of each dye in 100 ml PDB filled in 250 ml Erlnmeyer flasks. Two day old broth culture of each bacterium ($\times 10^{-6}$/ml) was inoculated separately in each flask and the inoculated flasks were incubated at 25 ± 1°C for 8 days in BOD incubator. Three flasks were kept for each treatment by keeping flasks devoid of bacterial culture inoculation as the control treatment. Decolourization was recorded like in case of earlier steps.

Temperature and pH optima of *B. licheniformis* for dye decolourization. pH range of 4.0 to 10.0 was used for studying the pH optima of Orange II sodium salt decolurization by the potential bacterium, *B. licheniformis*. Prepared broth was distributed in 250 ml Erlenmeyer flasks @ 100 ml each and sterilized at 15 p.s.i. pressure for 20 min. The 0.1 ml stock solution (1 g/10 ml) of the dye was added in 100 ml sterilized broth in flasks to give a final concentration of 100 ppm and 0.1 ml of two day old bacterial broth ($\times 10^{-6}$) was inoculated separately in each flask. Flasks devoid of bacterial broth but with PDB of different pH were kept as control treatments. Three replications were kept for each treatment and all flasks including control were incubated at 25 ± 1°C for next 8 days. Decolourization was recorded like in case of earlier steps.

Five different incubation temperatures (20, 25, 30, 35 and 40°C) were used for studying the temperature optima of Orange II Sodium salt decolorization with potential bacterium, *B. licheniformis*. Protocol for media preparation and setting up of experiment was same as was for pH studies, excepting medium pH, which was kept 7.2. Experiment was carried out in triplicate and all flasks including control were incubated at 5 different temperatures for next 10 days. Decolourization was recorded like in case of earlier steps.

Measurement of decolorization extent. Sample (3 ml) collected each time from each replication and centrifuged at 10000 rpm for 10 min was used for measuring decolorization

extent by measuring absorbance of supernatant at specific λ_{max} for each dye by using UV-Visible double beam Spectrophotometer (Unico-3802). Decolorization extent was calculated as:

Decolorization extent (%) = [100 x (OD_1-OD_t)]/OD_1

Where OD_1 is initial absorbance at 0 day, OD_t is absorbance after incubation for different periods under different experimental conditions, t is incubation time [20].

RESULTS AND DISCUSSION

Fungal and bacterial microflora in SMS of different mushrooms. Spent substrate from three different mushrooms varied both in their quantitative and as well as qualitative fungal population. SMS from *P. sajor-caju* harboured highest fungal population, dominated by *Aspergillus fumigatus*, followed by *Schizophyllum commune* and *Pezizomycotina* sp. (Table 2). SMS of *A. bisporus*, harboured next highest population exclusively dominated by *Aspergillus fumigatus*. SMS from *V. volvacea* although harboured three dominating fungi like *P. sajor-caju* SMS, but it exhibited two very distinct fungi (*Paecilomyces variotii* and *Pichia guilliermondii*) along with a common fungus (*A. fumigatus*). Although the information available on the subject is very scanty, however, in one report available; SMS from *A. bisporus* has been reported to be dominated by *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp. and *Glycodium* sp. [9]. Alike earlier study, the present study has also reported highest population of *Aspergillus fumigatus* in SMS of *A. bisporus* and *P. sajor-caju*.

Table 2: Fungal population in spent substrate of different mushrooms

Spent substrate	Fungus (5.8S rDNA sequencing)	CFU (×104)
A. bisporus	*Aspergillus fumigatus*	5.0
P. sajor-caju	*Aspergillus fumigatus*	8.5
	Schizophyllum commune	3.0
	Pezizomycotina sp.	3.0
V. volvacea	*Aspergillus fumigatus*	1.0
	Paecilomyces variotii	2.0
	Pichia guilliermondii	1.0

SMS of *P. sajor-caju* did harbour highest population as well as diversity of bacteria comprised of four different species (*B. licheniformis, Bacillus subtilis*, *Rummelibacillus stabekisii* and *Pseudomonas fluorescens*) compared with SMS of *A. bisporus* and *V. volvacea*, which harboured three and one species, respectively (Table 3). *V. volvacea* SMS harboured second highest bacterial population, dominated by only one species (*Bacillus pumilus*). Reports specifically on bacterial population dynamics in SMS of different mushrooms are not available in great numbers. However, in one report on *A. bisporus* SMS, a total 14 different operational taxonomic units have been assigned using 16S rDNA sequencing technique, out of which 12 were of Gram-positive bacteria, while rest two have link to *Comamonas* and *Sphingobacterium*. Among Gram-positive bacteria, the important were *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Brevibacterium*, *Arthrobacter*, *Microbacterium* etc. [21].

The present findings also prove the dominance of *Bacillus* sp. in SMS of *A. bisporus* and *V. volvacea*, while *Rummelibacillus stabekisii* and *Pseudomonas fluorescens* in *P. sajor-caju* SMS. The presence of *Sphingobacterium multivorum* in *A. bisporus* SMS has also been presented.

Table 3: Bacterial population in spent substrate of different mushrooms

Spent substrate	Bacterium (16S rDNA sequencing)	CFU ($\times 10^7$)
A. bisporus	*Bacillus subtilis*	9.0
	Bacillus pumilus	2.1
	Sphingobacterium multivorum	4.0
	Sphingobacterium multivorum	1.0
P. sajor-caju	*B. licheniformis*	0.64
	Bacillus subtilis	0.20
	Rummelibacillus stabekisii	14.9
	Pseudomonas fluorescens	10.0
	Pseudomonas fluorescens	10.0
V. volvacea	*Bacillus pumilus*	0.64
	Bacillus pumilus	29.0

Dye decolourization by different fungi. Out of seven fungi from SMS of three different mushrooms, highest decolourization of Chicago sky blue (95.0%) after 12 days of incubation was recorded with *Schizophyllum commune* isolated from *P. sajor-caju* SMS, followed by *Pezizomycotina* sp. again from the same SMS (Table 4). It was followed by three isolates of *A. fumigatus* isolated from three different SMSs (33.6 to 49.0%). Decolourization was completely absent in *Pichia guilliermondii* isolated from *V. volvacea* SMS. Decolourization increased with time of incubation and become near static after 10 days of incubation. In several earlier studies, researchers have reported the dye decolourization potential of microorganisms isolated from different ecosystems like soft wood chips [22], composting environment [23], dye house effluent [24], refuse dump soil [25] and Brazilian ecosystems [26] and have reported their relative importance in dye decolourization. Although the workers have emphasized the importance of microorganism from specific ecosystems but amongst fungi the potential dye decolourizers have been identified as mesophilic white rot fungi *Schizophyllum commune* [27-28] and *Aspergillus* spp. [29-30] similar to the present study.

Table 4: Decolourization of synthetic dye Chicago sky blue with different fungal isolates isolated from spent substrate of different mushrooms

Fungus	Dye decolourization (%) at different interval of time (days)						
	0-day	2-day	4-day	6-day	8-day	10-day	12-day
Aspergillus fumigatus (Ab)	0	6.7	22.9	28.7	30.3	31.8	33.6
Aspergillus fumigatus (Vv)	0	12.3	37.0	41.8	43.7	47.1	49.0
Paecilomyces variotii (Vv)	0	0	11.2	25.7	27.3	29.4	29.7
Pichia guilliermondii (Vv)	0	0	0	0	0	0	0.0
Aspergillus fumigatus (Ps)	0	11.0	32.1	36.0	34.9	36.2	40.5
Schizophyllum commune (Ps)	0	0	60.4	79.8	90.7	95.3	95.0
Pezizomycotina sp. (Ps)	0	0	53.5	54.7	73.8	87.8	92.0
$CD_{0.05}$	-	0.096	0.064	0.075	0.1405	0.039	0.081

The potential dye decolourizer fungus, *Schizophyllum commune* was further evaluated for its decolourization potential against 6 additional dyes for 18 day of incubation. This fungus exhibited significantly higher decolourization of Starch Azure (100%), followed by Reactive blue (92.5%), Rhodamine B (81.90%), Orange II sodium salt (73.40%) and Methyl Blue (67.8%). Negligible decolourizarion was recorded in case of Azure B (Table 5). Earlier studies

carried out on *S. commune* have also proved the dye decolourization potential of this fungus against Congo red [27] and Solar Golden Yellow R [28], a direct textile dye.

Table 5: Decolourization of different dyes by a promising fungus *Schizophyllum commune* isolated from oyster mushroom spent substrate

Dye	Dye decolourization (%) at different interval of time (days)						
	0-day	3-day	6-day	9-day	12-day	15-day	18-day
Methyl blue	0	0	15.9	29.6	46.2	67.2	67.8
Orange II Sodium salt	0	0	16.9	34.4	59.7	73.1	73.4
Rhodamine	0	0	13.6	14.3	18.6	27.8	81.6
Chicago sky	0	0	2.2	2.5	2.2	2.2	2.2
Azure B	0	0	4.1	3.4	4.1	4.0	4.0
Reactive blue	0	0	20.5	22.5	44.6	81.0	92.5
Starch Azure	0	0	100	100	100	100	100
$CD_{0.05}$	-	-	0.075	0.041	0.069	0.060	0.079

Dye decolourization by different bacteria. Out of eight different bacteria studied for their dye decolourization potential against Orange II sodium salt for 8 days of incubation, highest decolourization (66.1%) after 8 days of incubation was with *B. licheniformis* isolated from *P. sajor-caju* SMS, followed by *B. pumilus* (57.0%) isolated from *V. volvacea* SMS (Table 6). Lowest decolourization was with *B. subtilis* and *Rummelibacillus stabekisii* both isolated from *P. sajor-caju* SMS. The three isolates of *B. pumilus* isolated from SMS of three different mushrooms also varied in their dye decolourization potential. In majority of the dyes, highest decolourization was achieved up to 6 days of incubation, after which it remained almost static. Like in present study, the microorganisms from different ecosystems have also been tested earlier for their decolourization potential [22-26] and one aerobic non-filamentous bacterium from composting environment [23], *Clostridium bifermentans* from contaminated soil [7], *B. cereus* from dye house effluent [24] and *Micrococcus* sp. from refuse dump soil [25] have been reported to have significant dye decolourization potential.

Table 6: Decolourization of synthetic dye Orange II sodium salt with different bacteria isolated from spent substrate of different mushrooms

Bacterium	Dye decolourization (%) at different interval of time (days)				
	0-day	2-day	4-day	6-day	8-day
Bacillus pumilus (Vv)	0	0	48.4	57.7	57.0
Sphingobacterium multivorum-1 (Ab)	0	10	30.4	32.9	30.2
Bacillus pumilus (Vv)	0	22.0	28.6	44.7	45.9
Sphingobacterium multivorum-2 (Ab)	0	23.1	40.5	41.0	42.1
Rummelibacillus stabekisii (Ps)	0	12.7	15.9	11.6	10.0
Bacillus pumilus (Ab)	0	26.8	44.3	40.2	40.0
B. licheniformis (Ps)	0	17.3	17.8	62.7	66.1
B. subtilis (Ps)	0	1.1	1.3	1.4	0.0
$CD_{0.05}$	-	0.146	0.228	0.129	0.109

The two potential bacteria, *B. licheniformis* and *B. subtilis* isolated from *P. sajor caju* SMS were restudied from their decolourization potential against two more recalcitrant dyes (Azure B and Methyl blue), where in *B. subtilis* decolourized two dyes up to 44.6 and 91.3%, respectively. Decolourization of these two dyes was comparatively lower (15.0 and 75.8%) in case of *B. licheniformis* (Table 7). In most of the earlier studies, the dye decolourization potential of specific bacterium have been studied against several dyes and bacteria belonging to *Bacillus* spp. have been reported to have good decolourization potential [23-24].

Table 7: Decolourization of Azure B and Methyl blue with two potential bacteria *B. licheniformis* and *B. subtilis* isolated from SMS of *P. sajor-caju* under nutrient deficient conditions

Bacterium	Dye	Dye decolourization (%) at different interval of time (days)				
		0-day	2-day	4-day	6-day	8-day
B. licheniformis	Azure B	0	1.7	24.2	15.1	15.0
	Methyl blue	0	9.8	43.1	52.9	75.8
B. subtilis	Azure B	0	2.5	36.4	40.3	44.6
	Methyl blue	0	43.7	58.0	66.0	91.3
$CD_{0.05}$		-	0.045	0.064	0.194	0.113

pH and temperature optima for dye decolourization by *B. licheniformis.* The test bacterium was studied for its decolourization potential against Orange II Sodium salt at seven different pH levels, where in highest decolourization (85.6 and 81.1%) was recorded against pH levels of 6.0 and 7.0, followed by 8.0. Decolourization was almost similar at pH 5.0 and 9.0, while lowest at 4.0 (Table 8). The decolurization was almost negligible up to first 4 days of inoculation, which suddenly increased on day 6 of incubation.

Table 8: Effect of pH of the growing medium on decolourization of Orange II sodium salt with potential bacterium *B. licheniformis* isolated from spent mushroom substrate

pH	Dye decolourization (%) at different interval of time (days)				
	0-day	2-day	4-day	6-day	8-day
4.0	0	0	0	56.6	74.1
5.0	0	3	5.5	3.0	75.4
6.0	0	1.6	1.9	78.0	85.6
7.0	0	0	1.4	71.8	81.1
8.0	0	0	0	62.6	79.4
9.0	0	0	3.5	74.1	75.9
10.0	0	0	0	43.2	59.9
$CD_{0.05}$	-	0.026	0.053	0.089	0.064

Like pH, temperature of incubation has direct role is sustaining enzymatic activities and growth of different microorganisms in a cultural medium, which ultimately influences the dye decolourization process. The temperature optima of test bacterium for decolourization of Orange II Sodium salt was studied at 5 different temperatures. Highest decolourization (62.2% and 61.7%) was recorded at 25°C, closely followed by 20°C. The decolourization level decreased with increasing temperature of incubation (Table 9). Prior to this, several researchers have studied the pH and temperature optima of the microorganism of their interest and have reported wide range of pH (5-11) and temperature (20-40°C) for optimum decolourization by those

microorganisms [7, 31]. However, pH near 6-7 and temperature from 25 to 35°C have been reported more suitable for optimum decolourization. In literature, a vide variation in pH requirements for decolourization of different dyes through different microorganisms has been cited and it varied from 10 in case of *Clostridium bifermentans* SL186 for Reactive dyes [7], 7.0 in *Citrobacter* sp. CK3 for Reactive Red [32] and 5.0 in *Aspergillus niger* SA1 for Acid Red 151 [1]. Again for temperature, specific temperature has been indicated for decolorization of specific dye through a specific microorganism [1, 32]. However, unlike present study, the optimum decolourization of Reactive red has been cited through *Citrobacter* sp. CK3 at 32°C, while that of Acid red 151 through *Aspergillus niger* SA1 at mesophilic temperature (25-45°C) and Remazol brilliant blue R through *Streptomyces psammoticus* at 32°C. However, in none of the cases, the temperature optima of SMS inhibiting bacteria have been studied.

Table 9: Effect of temperature of incubation on decolourization of Orange II sodium salt with potential bacterium *B. licheniformis* isolated from SMS of *P. sajor-caju* under nutrient deficient conditions

Temperature	Dye decolourization (%) at different interval of time (days)					
(°C)	0-day	2-day	4-day	6-day	8-day	10-day
20	0	33.9	45.7	57.7	58.4	61.7
25	0	35.9	51.3	60.0	58.4	62.2
30	0	43.3	53.4	56.0	53.0	51.0
35	0	48.3	43.3	54.7	50.0	45.0
40	0	49.6	49.0	44.1	39.0	35.0
$CD_{0.05}$	-	0.149	0.071	0.067	0.194	0.081

In present study, variation in temperature optima for decolorization of Orange II sodium salt through *B. licheniformis* was mainly because of variation in bacterium and the dye used in study. Decolourization was higher at 25 and 20°C, because of 20-25°C is the optimum temperature for mycelial growth and fruiting of *P. sajor-caju*. Accordingly, the SMS of *P. sajor-caju* will also supports the microorganisms with their temperature optima in this temperature range. In few earlier studies, the decolourization has been reported at par at 15, 25 and 35°C, which is attributed to 15°C as the optimum temperature for fruiting of *A. bisporus* and *L. edodes* [33].

Proper disposal of SMS has always remained a challenging task for mushroom growers' as till now mushrooms are considered as prime product of mushroom cultivation and left over residual substrate, which contains a variety of nutrients and microbes is discarded unsystematically. Present study highlights both quantitative and qualitative variation in microflora of SMS from three different mushrooms and same has been proved earlier, as spent substrate from different mushrooms vary in their physico-chemical properties; hence also in inhabiting microbial population [31]. The study has also revealed higher population and more diversity of both fungi and bacteria in SMS from *P. sajor-caju*, which has again been proved in some earlier studies. Amongst the inhibiting microflora from SMS of different mushrooms, the fungus (*Schizophyllum commune)* and bacterium (*B. licheniformis)* have been found to have higher decolourization potential than others and like wise in earlier several reports, the SMS of oyster mushroom or the *Pleurotus* mycelium have been reported to have higher decolourization than other SMS or microbes from SMS of other mushroom [34]. *P. sajor-caju* is grown on pasteurized wheat straw/paddy straw substrate. Although, this mushroom has pH and temperature optima of 7-8 and 20-28°C, respectively for vegetative growth and fruiting, but its spent substrate also harbor other microorganisms of mesophilic nature, which have significant ligninolytic enzymes activity [10, 31] and contribute towards dyes decolourization [9].

CONCLUSION

Spent substrate from *P. sajor-caju* has been found to harbour highest and diverse population of both fungi and bacteria. Out of the isolated bacteria and fungi from SMS of three different mushrooms, the fungus (*Schizophyllum commune)* and bacterium (*B. licheniformis)* isolated from *P. sajor-caju* SMS, exhibited highest decolourization against different dyes. The pH and temperature optima for decolourization was recorded to be in the same range as was for the growth and fruiting of *P. sajor-caju*, which supports the suitability of oyster mushroom SMS as such in decolourization of different dyes.

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