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J. Environ. Biol.
32, 765-772 (2011)
ISSN: 0254-8704
CODEN: JEBIDP

Characterization and optimization of conditions for biodegradation of sella-rice mill effluent

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Publication Data

Paper received:
05 July 2010

Revised received:
18 November 2010

Accepted:
20 December 2010

Abstract

The sella-rice mill effluent is a major environmental pollutant requiring proper treatment before disposal. The present study has been conducted to isolate and characterize micro-organisms capable of growing on sella-rice effluent and to optimize conditions for its rapid bioremediation. Using three different types of media (LB, YEPDA and PDA), a total of 139 isolates were isolated from effluent samples collected from three different locations. Out of these, 45 isolates were found to utilize starch on starch medium, eight isolates showing high efficiency. For the optimization of conditions for maximum utilization of starch by selected isolates, parameters such as effect of addition of carbon and nitrogen sources, effect of growth factors, temperature and pH were studied. Maximum growth (absorbance of 2.10) and starch-utilization (varying in the range of 2.33 to 3.62) was observed on starch medium supplemented with peptone and yeast extract at 30°C with a pH of 6.0. These bacterial isolates also reduced the amount of starch (80.10%), BOD (64.24%) and COD (75.0%) of sella-rice mill effluent after 15 days of incubation. On the basis of morphological and biochemical characteristics, the selected isolates were found to belong to the genera *Lactobacillus* and *Micrococcus*.

Key words

Biodegradation, Sella–rice mill effluent; Starch-utilizing bacteria, Microorganism isolates

Introduction

In recent years, considerable attention has been paid to the industrial wastes, which are usually discharged on land or into different water bodies. At the rate the industrial development is going on in our country, the industrial activities will increase and would have an adverse impact on agriculture as well as on living organisms. This is likely to result in the degradation of environment (Chhonkar *et al.,* 2000). Various physico-chemical techniques have been studied for their applicability to the treatment of wastewater (Song *et al.,* 2004; Badani *et al.*, 2005; Rodrigues *et al.,* 2007), but several limitations in the physicochemical methods make the biological methods a favourable alternative for the removal of pollutants in the industrial effluents. The process of biodegradation is a well-established and powerful technique for treating domestic and industrial effluents (Collern, 2003). Microbial populations have an amazing and extensive capacity to degrade a variety of organic compounds (Atlas and Bartha, 1998).

Rice is one of the most important crops of the world. More than half of the world's population is dependent on rice. In India, rice is the prime cereal crop occupying an area of 42 million ha with an annual production of 76 million tons and contributing to nearly 42% of the country's food grain production (Rao, 2003). In Haryana, paddy is cultivated on 5.32 lakh ha of land in the districts of Karnal, Kurukshetra, Yamunanagar, Kaithal, Ambala, Sonepat, Panipat, Jind, Sirsa and Fatehabad. The total production per annum of rice in Haryana is 1.90 million tons (ASI, 2004). A major part of total rice products is converted into sella or parboiled rice, meaning "partially boiled" or partially cooked rice. It is economical as well as nutritious. Parboiling is a premilling process for paddy which originated in India (Subrahmanyan, 1971). Parboiled rice production generally requires

a large amount of water for soaking of the paddy. It has two simple steps *i.e.* soaking in hot water at 60-70°C for 3-3.5 hrs or steaming for 15-30 min (Paspia and Desikachar, 1980). After soaking, the water is drained out. This water if not properly treated could result in water pollution and odour nuisance to residents. Water pollution can be caused by high levels of organic material present in wastewater (Manogari *et al*., 2008). The volume of effluent generated from sella-rice mill is approximately 900-1000 l ton^{-1} of paddy (Paspia and Desikachar, 1980). This effluent has high BOD, COD and organic contents mainly in the form of starch, thus having the potential to damage and deteriorate the environment (Pradhan and Sahu, 2004). Therefore, it needs to be treated before disposal. Literature reports indicate that biodegradation involving microorganisms is a suitable process for industrial wastewater treatment (Noorjahan *et al.,* 2005). Hence, in the present study, an attempt has been made to develop a process for biodegradation of sella-rice mill effluent using microorganisms isolated in the laboratory.

Materials and Methods

Collection of samples, physico-chemical analysis and isolation of bacteria: Samples (5 l) of sella-rice mill effluents were collected in presterilized glass bottles from three regions in Haryana, *i.e.*, Fatehabad, Jind and Yamunanagar. From each region, the samples were collected from two different sites: (i) Directly from the boiler or the outlet, and (ii) the mud/soil/effluent from the field where the effluent is disposed off. The pH, biological oxygen demand (BOD) and chemical oxygen demand (COD) of the samples was determined within 48 hr and they were then stored in the refrigerator at 7-8°C for further chemical (suspended solids, dissolved oxygen, starch and nitrogen content) analysis.

For obtaining microorganisms capable of degrading sella-rice mill effluent, starch utilizing bacteria were isolated. Aliquots of 0.1 ml of samples were spread on three different media, *viz*., Luria broth (LB), potato dextrose agar (PDA) and yeast extract peptone dextrose agar (YEPDA) and the plates were incubated at 30°C for 48 hrs. For enrichment, 1 g soil sample collected from field pit soil was suspended in 100 ml of unsterilized effluent and incubated at 30°C under stationary conditions for 48 hr, after which 0.1 ml of appropriate dilutions were spread on three media. Well isolated colonies showing different colony morphology were selected and transferred on slants. All the isolates were spotted on solid starch medium (soluble starch-20.0 g l^{-1}, yeast extract-5.0 g l^{-1} and peptone-5.0 g l^{-1}, pH- 6.0) and the plates were then incubated. Starch utilization by the isolates was determined by looking for zone formation after flooding the plates with iodine solution. Isolates showing hollow zone around the colonies were selected and their starch utilizing efficiency was calculated by using the following equation: Efficiency (cm) = Z+C/C, where Z=Radius of hollow zone, and C=Radius of colony. The colonies with high efficiency were selected and preserved for further studies.

Optimization of conditions: The microorganisms capable of starch utilization were isolated and selected. The effect of addition of nutrients, *viz*., 1.0% glucose (G), 0.1% yeast extract (YE), 0.1% peptone (P) and 0.2% ammonium sulphate (AS) was studied using eight selected isolates on solid starch agar medium supplemented with eight different combinations (Starch alone; Starch+P; Starch+P+YE; Starch+AS; Starch+G; Starch+G+AS; Starch+G+YE; and Starch+G+P) of carbon and nitrogen sources. The plates were incubated at 30°C for 48 hr, zone formation was observed and starch utilization efficiency was calculated. For studying the effect of addition of nutrients in the broth, 10 ml inoculum of each selected isolate was added to 250 ml of starch broth supplemented with the same eight combinations of nutrients. Starch broth without any additional nutrients served as the control. All the flasks were incubated at 30°C under shaking conditions for 48 hr. Samples (10 ml) were withdrawn aseptically at intervals of 0, 4, 8, 12, 20, 24, 28, 32, 36, 44, and 48 hr and centrifuged at 10,000 rpm for 15 min. The supernatant of each isolate was taken for estimation of utilized starch using standard method (Malik and Singh, 1980) and the pellet obtained after centrifugation was resuspended in distilled water and absorbance was measured at 600 nm.

The effect of temperature and pH on the utilization of starch was examined by growing all the eight selected isolates in three different types of media (Starch alone, Starch+G+YE, and Starch+P+YE) at 25, 30 and 35°C and in pH ranging between 6.0 to 8.0. Samples were incubated under shaking conditions for 48 hr. Samples were withdrawn aseptically at 4 hrs intervals for 48 hr and processed for determination of starch of the supernatant and for recording absorbance at 600 nm of the cell suspension.

Screening of starch-utilizing-bacteria: To examine the practical application of the selected isolates, out of eight isolates, four were further selected for checking degradation of sella-rice mill effluent under laboratory conditions. One litre of sella-rice mill effluent, either supplemented with nutrients (starch-5.0%, YE-0.1% and peptone-0.1%) or without nutrients, was inoculated with 1.0% inocula of each of the four isolates and the flasks were incubated at 30°C for 15 days. Samples (20 ml) were withdrawn at time intervals of 0, 1, 3, 9 and 15 days, centrifuged at 10,000 rpm for 15 min and the supernatant was analyzed for reduction of starch, pH, BOD (APHA, 1995) and COD (HMSO, 1986).

Characterization of efficient bacterial isolates: Four selected isolates were studied morphologically by microscopic examination (Gram's reaction, cell shape and arrangement, spore formation *etc.*), and identified and characterized on the basis of standard biochemical tests (Cappuccino and Sherman, 1983). Twelve biochemical tests (indole production, methyl red reaction, voges proskauer test, catalase test, gelatin liquefaction, starch hydrolysis, nitrate reduction, citrate utilization, urease production, sugars fermentation, oxidase test and esculin hydrolysis) were performed for identification of bacterial isolates. Utilization of various sugars (glucose, mannitol, lactose, xylose, maltose, sucrose, fructose, galactose, inositol and cellobiose) was tested by using basal medium (ammonium sulfate = 1.0 g l^{-1}, NaCl = 0.2 g l^{-1}, $MgSO_4$ = 0.2 g l^{-1}, yeast extract = 0.2 g l^{-1} and 4.0 ml of 0.2% bromocresol purple, pH = 7.0). For determination of gas

production, each sugar (1.0%) was added into a test tube containing a Durham's tube in inverted position. The tubes were inoculated with different isolates and incubated at 30°C for 48 hrs. Observations were recorded for change in colour and gas production.

Results and Discussion

Physico-chemical analysis of sella-rice mill effluent: It was observed that the colour of the effluents was yellow brown, the odour was unpleasant and the effluents were turbid. The pH of the effluents varied from 6.89 to 7.36. The suspended solids were in the range of 432 to 541 mg l^{-1}. The major organic matter of the effluents was starch that varied from 700 to 1200 µg m l^{-1} in samples. The COD of effluents was recorded in the range of 1400 to 2200 mg l^{-1} while the BOD varied from 360 to 480 mg l^{-1}. The dissolved oxygen and nitrogen contents of sella-rice mill effluents were found below 4.7 to 5.6 mg l^{-1} and 0.0084 to 0.0252%, respectively. In a similar kind of study, the detailed process and effluent characteristics of a rice mill were studied by Pradhan and Sahu (2004) in Sambalpur district in

Table - 1: Effect of addition of nutrients (on solid starch medium) on starch utilization efficiency of selected isolates.

Media	Starch utilizing efficiency (cm)							
(Nutrients)	LB-10S	LB-9S	PA-20M	Y-3M	LB-18F	Y-8F	LB-17F	Y-11M
Control	2.15	1.42	2.00	2.33	1.66	1.75	2.12	1.81
S + P	2.00	2.62	2.10	2.42	1.80	2.33	1.80	2.00
S+P+YE	2.41	2.85	3.52	3.62	3.33	3.36	2.33	2.81
S+AS	2.05	1.33	1.90	2.00	1.53	2.10	2.00	1.64
S+G	2.20	1.20	1.87	1.90	1.33	2.10	1.90	2.00
S+G+AS	2.10	1.10	1.89	2.01	1.50	1.63	2.11	1.93
S+G+YE	1.83	2.21	1.20	2.40	2.40	2.60	2.50	2.32
S+P+G	2.62	1.87	2.33	2.00	1.75	1.66	2.42	2.30

S = Starch (1.0%); G = Glucose (1%); AS = Ammonium sulphate (0.2%); P = Peptone (0.1%), Y.E. = Yeast extract (0.1%), *Values are mean of three replicates

Table - 2: Effect of temperature and pH on starch utilization after 48 hrs.

Isolates	Starch utilization (µg ml^{-1})					
	Temperature (°C)			pH		
	25	30	35	6.0	7.0	8.0
LB-9S	8500	3300	7000	3500	8700	8900
LB-10S	8700	4500	8900	3500	9000	9000
LB-17F	8900	5100	9500	4200	8800	8900
LB-18F	8900	3300	7200	5800	9000	9000
Y-3M	9000	3500	8200	3600	8800	8900
Y-8F	9200	5900	6200	5000	9100	9200
Y-11M	8200	3700	7100	3500	9000	9100
PA-20M	9100	5000	5200	3500	8700	8800

*Values are mean of three replicates

Table - 3: Effect of temperature and pH on growth (OD at 600 nm) of selected isolates

Isolates	Absorbance at 600 nm					
	Temperature (°C)			pH		
	25	30	35	6.0	7.0	8.0
LB-9S	0.42	1.84	1.37	1.89	1.16	1.0
LB-10S	0.95	1.89	1.30	1.87	0.78	0.64
LB-17F	0.37	1.82	1.33	1.84	1.19	1.07
LB-18F	1.12	1.99	1.35	1.44	0.97	0.25
Y-3M	0.27	1.85	1.58	1.88	1.0	1.0
Y-8F	0.11	1.65	1.48	1.70	1.56	1.43
Y-11M	1.10	1.99	1.73	1.89	0.68	0.65
PA-20M	0.19	1.99	1.33	1.85	1.51	1.60

*Values are mean of three replicates

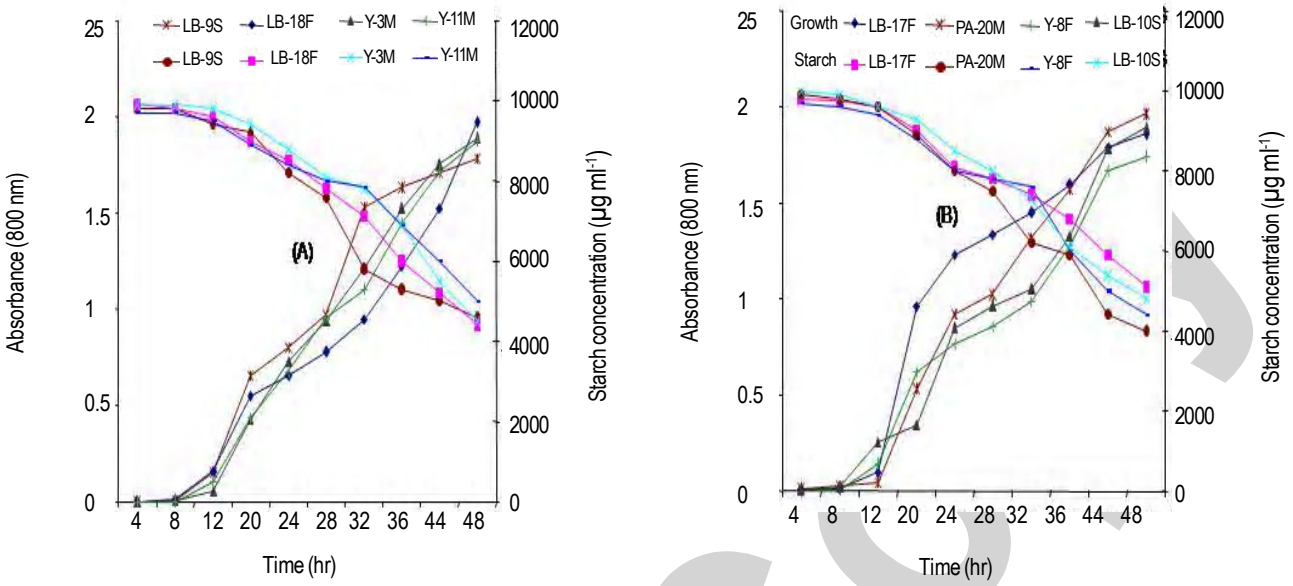

Fig. 1: Growth (increase OD at 600 nm) (A) and Utilization of starch (decrease concentration of starch) (B) by different bacterial isolates in media containing Starch (1.0%) + Peptone (0.2%) + Yeast extract (0.2%) after 48 hrs

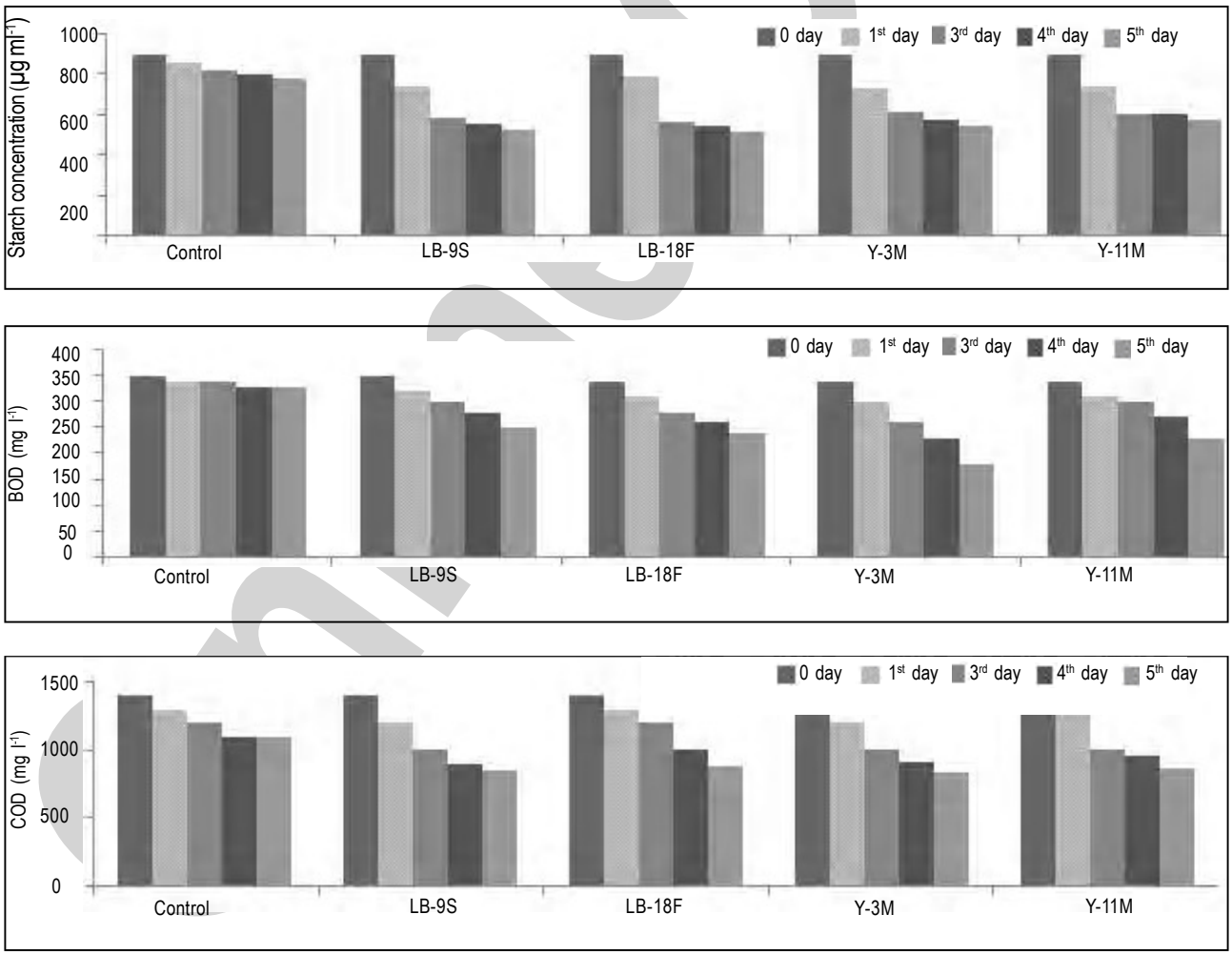

Fig. 2: Reduction of starch, biological oxygen demand (BOD) and chemical oxygen demand (COD) of sella-rice mill effluent by using selected bacterial isolates (without nutrients)

Orissa. Comparable to the results obtained in the present study, the physico-chemical characteristics of the effluent revealed an alkaline pH (8.0), with a low concentration of dissolved oxygen (0.9 mg l^{-1}) and total suspended solids (530 mg l^{-1}) and moderate concentrations of COD (640 mg l^{-1}) and BOD (450 mg l^{-1}). On the other hand, Amathussalam and Gnanaganesan (2004) conducted studies on tannery effluent and reported total dissolved solids to be in the range of 1936 mg l^{-1}, with high BOD (8739 mg l^{-1}) and COD (21965 mg l^{-1}) values. Similarly, BOD and COD of spent wash from distillery have also been reported to be quite high, 50000 and 110000 mg l^{-1}, respectively, (Mohana *et al.,* 2009). Thus, literature reports on physico-chemical analysis of industrial effluents reveal that sella-rice mill effluents possess low BOD, COD and organic matter in comparison to effluents generated by other industries, however, in quantitative terms it compels for treatment before disposal.

Isolation of starch-utilizing-bacteria: On the basis of differences in colony morphology, a total number of 139 isolates were purified. Colour of the colonies varied from colourless to white to yellow and the size varied from small, pinhead to large and spreading. Growth when checked on solid starch medium resulted into categorization of the isolates into two groups. Out of 139 isolates obtained, 45 were found to form zone of clearance indicating utilization of starch while other isolates were found to be starch negative. Eight isolates (LB-10S, LB-9S, PA-20M, Y-3M, LB-18F, Y-8F, LB-17F, Y-11M) were found to be better starch-utilizers as compared to others. Although several studies have been conducted on isolation of bacteria from industrial effluents, but there are only limited reports on isolation of starch-utilizin-bacteria. In one such study, Ayyasamy *et al.* (2008) reported isolation of starch degrading bacteria from sago industry effluent and effluent contaminated soil.

Effect of addition of nutrients: It was found that out of the eight different combinations tested, starch agar containing peptone and yeast extract best supported the growth of most of the isolates. On this medium, all the isolates also showed high starch utilizing efficiency varying in the range of 2.33 to 3.62 (Table 1). The growth and utilization of starch by eight selected isolates was further checked in starch broth supplemented with the same eight combinations of carbon and nitrogen sources. It was found that there was no utilization of starch as also supported by the absence of growth of any of the selected isolates in starch broth. However, addition of glucose, ammonium sulphate and peptone when taken alone supported the growth of all the eight isolates as determined by increase in absorbance, but there was negligible decrease in the concentration of starch after 48 hr of incubation. When the effect of addition of yeast extract as a source of growth factor was studied in combination with glucose and peptone, it was found that supplementation of yeast extract along with glucose resulted in an increase in growth (from an absorbance of 1.2 to 1.6) and a slight reduction in starch (from 9900 to 7000 µg ml^{-1}). However, best results were obtained upon supplementation of the starch broth with yeast extract along with peptone. The amount of starch decreased from 9900 to less than 4000 µg ml^{-1} by all isolates. Growth of all the isolates was also more, depicting an absorbance around 2.10. Maximum decrease in the amount of starch was observed in isolates PA-20M, Y-3M, LB-18F and LB-9S (Fig. 1). The effect of addition of glucose and peptone in the absence of yeast extract showed that although there was good growth of all the cultures, but the decrease in the amount of starch was only slight. Therefore, the best combination which resulted in good growth as well as maximum starch utilization was concluded to be yeast extract along with peptone.

Effect of temperature and pH: At all the three temperatures and pH, the selected isolates showed very slow growth and low starch utilization when grown in starch broth alone. When the starch broth was supplemented with glucose and yeast extract, the growth of all the isolates was slightly better and maximum utilization of starch was observed in presence of peptone and yeast extract at 30 and 35°C with pH 6.0; however, there was still very slow growth at 25°C and pH 8.0. Isolates LB-9S, LB-18F, Y-3M and Y-11M showed maximum reduction of starch (from 9900 to 3300 µg ml^{-1}) and good growth at 30°C and pH 6.0 (Table 2,3).

In most of the studies involving isolation of microorganisms from industrial effluents, optimization studies have been conducted. More recently, Kaushik and Thakur (2009) isolated 5 different fungi from distillery mill site and optimized process parameters for decolorization by using different factors like addition of growth factors, duration, pH, temperature, and inoculum size. Maximum color (38 to 62%) was removed at pH 3, temperature 30°C, and upon supplementation of dextrose (0.05%) and sodium nitrate (0.025%) by all the fungi. In another study, it was reported that a medium containing 2, 4, 6-trichlorophenol (2, 4, 6-TCP) as a sole carbon source supplemented with 1% glucose at pH 7 and 30°C stimulated the growth of *P. fluorescens* and enhanced the ability to utilize phenol from industrial effluent after 40 days (Lin *et al.,* 2008). Similarly, various parameters were studied for standardization of maximum growth and colour reduction by bacterial strains isolated from pulp and paper mill effluent (Mishra and Thakur, 2010). Maximum reduction in color (85%) and COD (90%) and lignin removal (25-69%) was observed at pH 8, temperature 35°C, and sucrose (2.5%) supplementation after 48 hrs. Singh *et al.* (2007) while studying microbial decolorization and bioremediation of melanoidin containing molasses spent wash, reported better decolorization and COD reduction under optimal conditions (supplementation of 0.5% peptone and 10% glucose at pH 7), and therefore concluded that the decolorization and COD reduction activity of microorganisms is also regulated by the addition of carbon and nitrogen sources in the basal medium. Thus, standardization of various parameters is a prerequisite for using microorganisms to their maximum potential for bioremediation purpose.

Degradation of sella-rice mill effluent: In the absence of nutrients, all the four isolates reduced the amount of starch present in the effluent from 900 to approximately 550 µg ml^{-1} after 15 days of incubation on rotary shaker. Likewise, there was slight decrease in pH, BOD as well as COD (Fig. 2). But in the presence of nutrients,

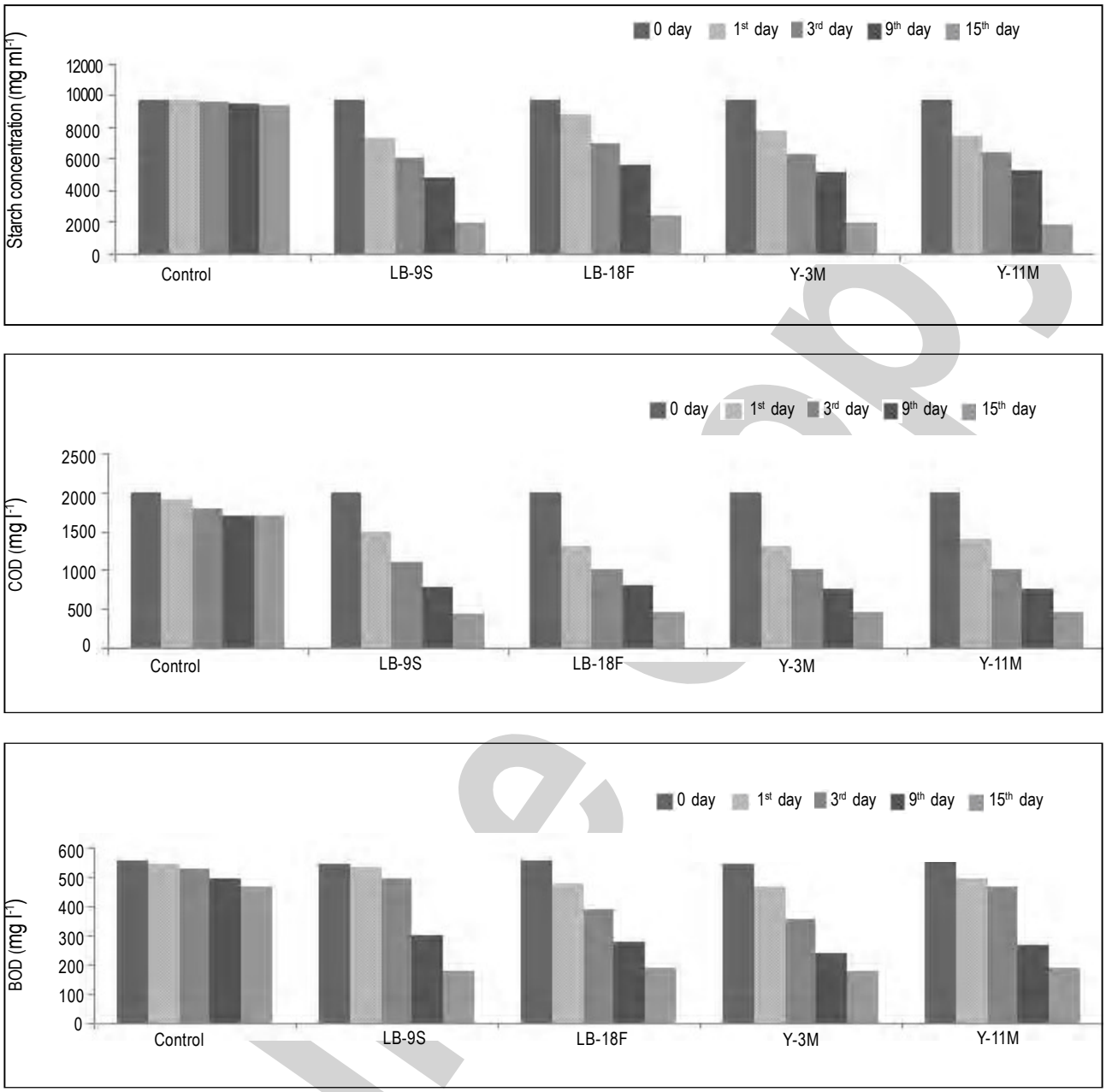

Fig. 3: Reduction of starch, chemical oxygen demand (COD) and biological oxygen demand (BOD) of sella-rice mill effluent by using selected bacterial isolates (with nutrient)

all the four isolates were found to be growing better and also reduced the amount of starch from 10,100 to 2000 µg ml^{-1} (80.10%), BOD and COD varied from 560 to 200 mg l^{-1} (64.24%) and from 2000 to 500 mg l^{-1} (75.0%), respectively, (Fig. 3); and the pH of effluent decreased from 6.0 to 4.7 after 15 days. In addition to reduction in starch, pH, BOD and COD; incubation of the effluents in presence of these isolates also resulted into an improvement in the colour and smell of the effluent. After 15 days of incubation, the colour of the effluent changed from dark brown to light brown and the foul smell also became pleasant/bearable (Fig. 4). Similar results were also reported by Ayyasamy *et al.* (2008) who reported 85% degradation of starch in sago industry effluent by using a bacterial consortium composed of *Alcaligenes, Bacillus* and *Corynebacteria* isolated from the effluent. In another study, Singh *et al.* (2007) studied microbial decolorization and bioremediation of melanoidin containing molasses spent wash and found that *Bacillus* sp. isolated from soil was capable of removing COD (85.35%) and colour (81.10%) from the distillery waste after 9 days. Manogari *et al.* (2008) studied biodegradation of rice mill effluent by using free and immobilised cells of *Pseudomonas* sp. cells in a packed bed system. The reduction in the COD and BOD was found to be 86.44 and 55.34% respectively, after 24 hr. A comparative study on the potential use of free cells and immobilized cells of

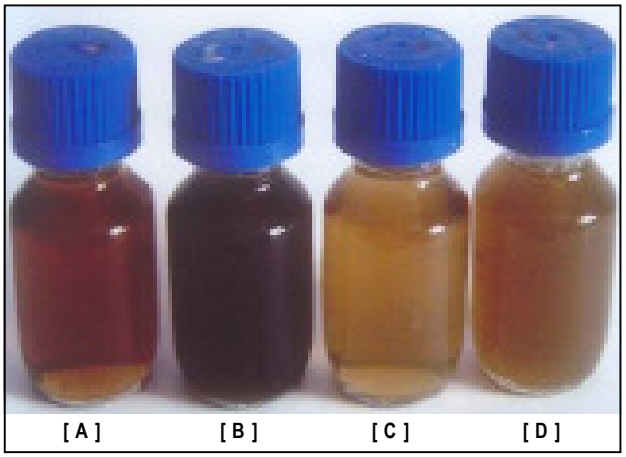

Fig. 4: Effect of inoculation (with isolate number LB-9S) on bioremediation of sella-rice mill effluent, (A) Unioculated: Supernatant obtained after centrifugation after 15 d of incubation (B) B-Uninoculated: Sella-rice mill effluent after 15 d of incubation (C) C-Inoculated (LB-9S): Supernatant obtained after centrifugation after 15 d of incubation (D) D-Inoculated (LB-9S): Sella-rice mill effluent after 15 d of incubation

Flavobacterium sp. has been reported with reduction of 66.4% of total dissolved solids, 35.9% BOD and 87% COD in treatment of tannery effluent *(*Elangovan *et al.,* 2002). In another study carried out by Abubacker *et al*. (2003) with tannery effluents, bacteria such as *Bacillus subtilis* (NCBT 011), *Citrobacter* (NCBT016) and *Proteus* (NCBT 018) were found to play a role in the reduction of pollutants in 48 hr. Srividya and Kanmani (2010) found that the *Pseudomonas fluorescens* and indigenous microorganisms were responsible for bioremediation of nickel contaminated soil. Thus, a large number of studies report the utilization of microorganisms for removal of pollutants from several industrial wastes, but studies on isolation of effective starch-utilizers are still limited.

Characterization and identification of selected bacterial isolates: Selected four bacterial isolates (LB-9S, LB-18F, Y-3M and Y-11M) were Gram - positive and non-spore formers. Most of them were found to be negative for methyl red and indole production test. These cultures did not hydrolyze gelatin but used esculin and starch. 2 isolates, *i.e*., LB-9S and Y-3M could use citrate as sole source of carbon for their growth and were found to be positive for nitrate reduction, whereas the other 2 isolates (Y-3M and Y-11M) were catalase positive and oxidase negative. On the basis of their characteristics features, the isolates were found to be belonging to the genus *Lactobacillus* and *Micrococcus*.

The present study provides important baseline information for the use of microorganisms for the degradation of sella-rice mill effluent. The study has resulted in the isolation of four very efficient bacterial strains possessing high starch-utilizing efficiency, suggesting a potential role for exploitation of these isolates in bioremediation of such effluents.

Acknowledgments

The authors wish to thank Dr. B.S. Kundu, Head and Professor, Deptt. of Microbiology, for his valuable suggestions and support during this work.

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