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Isolation and Optimization of Tobacco Decomposing *Bacillus* and *Lactobacillus* Sp.

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ABSTRACT
Three bacteria from a tobacco industry effluent and one from a sewage sample were isolated on a medium comprising of 1% tobacco powder as sole source of nutrients. Bacteria isolated from the industrial waste water were identified as *Bacillus cereus*, *B. alvei* and *B. circulans*. While, *Lactobacillus* sp. was isolated from the sewage sample. The bacteria were optimized for various growth conditions in a medium comprising of aqueous extract of 1% tobacco powder in MIII. Provision of 0.1% molasses (MIII) enhanced growth of *B.cereus* as compared to its cultivation in MII. Growth of *B.cereus* up to two weeks in molasses supplemented 1% tobacco powder (MIV) medium resulted into retroprogressive decreases in total suspended matter, ash content and total organic matter. So that at the last sampling period these parameter reduced up to 92%, 82% and 42%, respectively as compared to control values. The bacterial isolates in general and the *B.cereus* practically appear promising for designing *in situ* biotreatment plants for tobacco processing units and are likely to add in reducing environmental contamination originating from tobacco allied toxic compounds.

Keywords: Tobacco effluents' biotreatment; Tobacco detoxifying bacteria; Water pollution.

INTRODUCTION

Tobacco is cultivated in Pakistan at relatively large scale. The leaves are processed both by household level small industries as well as various tobacco companies for making cigarettes, in addition to some other products of local use. Rejected portion of tobacco leaves as well as effluents of tobacco industries is major wasteof such tobacco processing units and plants. Disposal of these pollutants to a natural water system not only increases its organic load accompanied by higher biological oxygen demands, but its toxicity due to the presence of alkaloids, nicotine and tannins may influence the aquatic biota negatively (Meher *et al*.1995; Sax & Lewis 1989; Saunders & Blume 1981).

Tobacco wastewater furthers the decline in normal aquatic life including fishes. Therefore, treatment plants for decomposing tobacco remnants are required by tobacco factories. Owing to the established toxicity of tobacco-related effluents a number of waste water treatment processes have been described (Bejankiwar 2002; De Lucas *et al*. 1998; Civilin *et al*. 1997). Denicotinization of the effluents can be done with physio- chemical treatments (Ireland *et al*.1980; Clarke & Stanely 1964) but alternatives involving biological methods appear environmentally friendly.

Here microorganism's ability to grow and destroy nicotine and alkaloids is exploited (Gravely *et al*.1977). However, it is known that shock inputs and overloading of biological treatment plants often inhibit bacterial activity and exert toxic effects on various micro-organisms employed in the biological treatment plants. (Sponza 2002; Munari 1986 ;Saunders & Blume 1981).Therefore, isolation of bacteria which can withstand varying levels of the pollutants as well as are capable of degrading tobacco biomass is imperative for designing reliable *in situ* tobacco biodegradation treatment plants. For the present study tobacco wastes in the form of effluent as well as dried processed leaves were obtained from a cigarette making industry. The effluent and a sewage sample were processed for the isolation of tobacco degrading bacteria. Isolation medium com-

prised of 1% powdered tobacco. In this report a bacterium has been worked out which can specifically degrade tobacco industries' effluent containing various amounts of the waste and thus reduces organic load of the treated effluent. Minimizing untreated tobacco waste load and the biochemical oxygen demand of recipient waters may prove a positive step towards rehabilitation of the aquatic biota.

MATERIALS AND METHODS

Four tobacco degrading bacteria were isolated from tobacco and sewage waste waters on a selective medium designated as MI that contained 1% tobacco powder and 1.5% agar in distilled water. The effluents were collected from a cigarette making industry and a sewage holding pond. Each of the effluents, 0.1ml was spreaded evenly on MI agar plates. Morphologically three different colonies obtained from tobacco waste water and one from the sewage sample were processed for pure culturing. The bacterial isolates were characterized and identified according to the methods of Konenan *et al*.(1997) and Holts *et al*.(1994)

Another medium, MII was prepared by boiling 1gm of dried tobacco powder in 100ml of water for ½ an hour in a reflex condenser. It was then filtered through Whatman filter paper No. 1 followed by centrifugation at 4000 rpm for 15 minutes. The supernatant served as MII. For optimization of growth conditions the bacterial isolates were inoculated in freshly prepared MII media under different experimental conditions. All experiments were performed in triplicates and each replicate contained 5 ml of inoculated medium. Bacterial growth was assessed at pH 5, 7 and 9. They were then grown at room temperature (25^{o}C ± 1), 37^{o}C and 50^{o}C at their corresponding pH optima. The growth was also checked on orbital shaker at 120 rpm. To optimize inoculum size bacterial growths were initiated with 1%, 5% and 10% inocula. Growth in all these experiments was measured by taking optical density(O.D.) of the bacterial cultures at 600nm after 24 hours of incubations.

Two of the bacterial isolates were cultivated in media MII and MIII under their optimum growth conditions. The latter medium contained 0.1% molasses in MII. Growth as well as pH of MIII were monitored daily up to 4 days post inoculation. Then up scaling of the isolate designated as Na-C was done in another medium, MIV under its optimum growth conditions. MIV was prepared like that of MII, except that the suspended tobacco material was retained. Finally four liters of the culture was raised in an aspirator under optimum conditions of temperature and aeration. Samples were taken daily starting from 6th day of inoculation up to the completion of 9th day. Each sample, 5ml was introduced in a dried pre-weighed crucible, in triplicates. The crucibles were then kept in an oven at 105^{o}C for 24 hours. After recording their weights they were kept in furnace at 400^{o}C for 4 to5 hours. Again their weights were determined. From these values total suspended material, total organic and total ash contents were determined according to Gupta (2000). Well shaken uninoculated MIV medium was similarly processed for the estimation of control values of these parameters. Statistical comparisons were made by employing Student's-test and single factor analysis of variance (Campbell 1989).

RESULTS AND DISCUSSION

The bacterial isolates were found motile and positive and negative for catalase and "Voges proskuer" tests, respectively. They vary in other characteristics as shown in Table 1. Based upon their characteristics, the isolates designated as Na-A, Na-B, Na-C and Na-D were tentatively identified as *Bacillus alvei, B.circulans, B.cereus* and *Lactobacillus* sp., respectively**.**

The isolates, Na-A and Na-B grew best at initial pH 5.While, they showed comparable growth when incubated at 37^{o}C and 50^{o}C. The other two bacterial species preferred alkaline pH and 37^{o}C incubation temperature as shown in Table 2. Aeration increased growth of *B.cereus* and *Lactobacilli* sp. significantly. One and 10% inocula appeared optimium for *B.alvei* and *Lactobacilli* sp., respectively Table 2.

When the two isolates were cultivated in MII and MIII, it appeared that provision of 0.1% molasses significantly increased growth of *B. cereus,* so that after 24 hours of incubation O.D of MIII culture turned out to be 0.147 ±0.048 as compared to the value of 0.085 ± 0.62 for MII. The *Lactobacilli* sp. remained indifferent to the presence or absence of molasses and the O.D. values appeared as 0.493 ±0.05 and 0.463 ± 0.02 in the media MIII and MII, respectively. When these bacteria were incubated up to 4 days, it appeared that *B .cereus* grew progressively accompanied in general with increases in pH (Fig.1a). Whilst in case of *Lactobacillus* sp. after two days of incubation growth decreased with rise in pH levels (Fig.1b). Up scaling of *B.cereus* in MIV was not studied for growth. However, analysis of the samples in terms of total suspended matter, total organic and ash

contents expressed a general retrogressive decrease throughout the period studied (Fig .2).

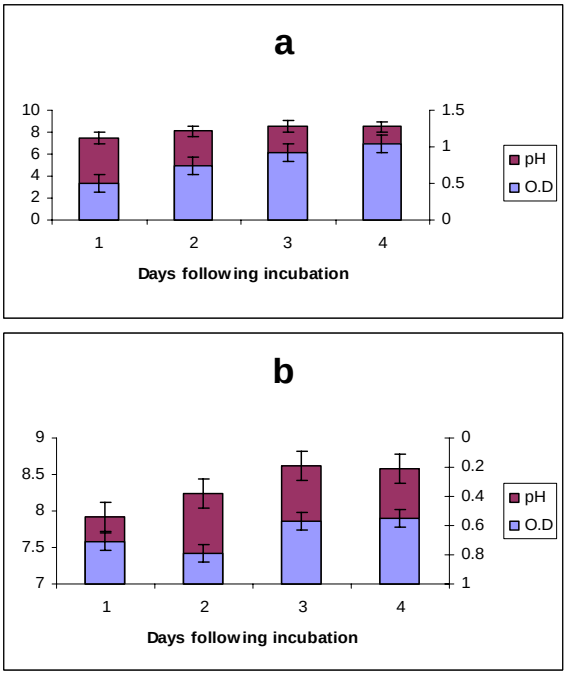

Figure1. Post-incubation growth and accompanying pH levels of *Bacillus cereus* (a) and *Lactobacilllus* sp. (b) in MIII medium at their corresponding optimum conditions.

Uninoculated MIV had about 1120, 680 and 460 mg/100ml of total suspended matter, total ash content and total organic content, respectively. *B.cereus* caused 82.14%, 61.76% and 56.521% decreases in this parameter, respectively at second sampling period.

The corresponding values at the last study period turned out to be 64.29%, 82.35% and 42.72%. About 13% increase in total organic matter content of the experimental MIV medium at 1st sampling point of incubation could be attributed to formation of bacterial biomass which then appeared to utilize the remaining organic matter throughout the study period. Results of the present work show potential of the reported bacterial isolate to degrade tobacco contents of industrial effluents.

Large quantities of tobacco waste water are generated during processing and cigarette making and these wastes may exert toxic effects due to presence of alkaloids, nicotine and tannins (Meher *et al*. 1995; Sax & Lewis 1989).Nicotine derived nitrosamines represent important tobacco carcinogens present in tobacco wastes (Gorrod and Jacob 1999).In this context it is alarming that the effluent has been introduced ultimately to natural water system without any biological treatment in the study area. Cumulative effects concerning any use of such contaminated waters both for humans as well as animals might be speculated.

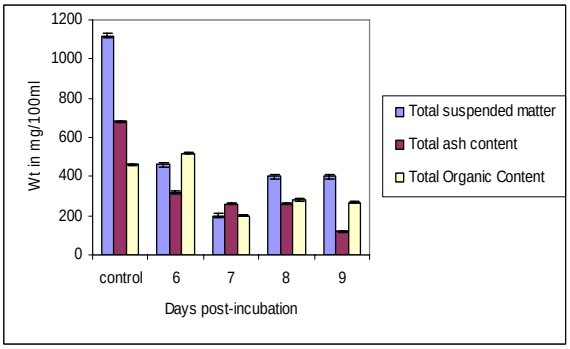

Figure 2 . Total suspended matter, total ash content and total organic content of control and inoculated with the *B.cereus* in MIV medium at various sampling periods.

Decreases in the total organic load following growth of *B.cereus* in MIV medium is promising in designing *in situ* biotreatment plants for the tobacco processing units. Provision of minute quantity of molasses which is a sugar industrial waste would hasten the bioremediation process. Alkaliphilic nature of the bacterium may prove useful in keeping away contamination of a biotreatment plant by less useful microbial agents at start or a subsequent phase of effluents loadings.

However, aeration would be required for this isolate. Toxic compounds in tobacco wastewater have been shown detrimental for microbial activities in biological treatment units. For instance, Sponza (2002) has described nicotine, flavoring chemicals containing glycogen and alcohols, absorbable organic halogens and pesticides from tobacco leaves as most important sources of contamination of tobacco industry waste water.

The author also described that input of waste water containing such toxic substances caused decreases in bacterial numbers and reduced the treatment efficiency in treatment plant. Sponza (2002) reported that total and fecal coliform bacterial profiles, fluctuations in biological treatment unit and a known/unknown source of toxicity can inhibit the microbial growth. In this regard bacterial species worked in this investigation appear important. As they grew in presence of tobacco as sole or major source of nutrients. Albeit detailed studies involving analysis of chemical content of tobacco waste water before and after the bacterial growth are required to be performed.

However, %reduction in total organic content following 2-week growth of *B. cereus* in MIV is suggestive to speculate lowering of BOD of the "treated effluent".

Table 1: Physio-biochemical characteristics of the bacterial isolates and their identification ∗.

Isolate code.	Cell morphology	Endospore	Oxidase *(Hemolytic activity)*	Lectithinase *(Nitrate reduction)*	Indole production *(Citrate utilization)* .	Acid from glucose	Result
Na-A	*Gram +ve Bacilli 3.25 x 2.62*[a]	*Elliptical 2.1 x1.05*	*– ve (– ve)*	*– ve (– ve)*	*+ ve (-ve)*	*– ve*	Bacillus alvei
Na-B	*Gram +ve Bacilli 2.1 x 0.735*	*Elliptical 2.1 x1.05*	*– ve (– ve)*	*– ve (+ ve)*	*– ve (-ve)*	*– ve*	Bacillus circulans
Na-C	*Gram +ve Bacilli 4.09 x 0.945*	*Oval to Elliptical 2.1 x1.26*	*+ve (– ve)*	*+ve (– ve)*	*N.R (N.R)*	*N.R*	Bacillus cereus
Na-D	*Gram +ve Coco bacilli 3.15x1.165*	*– ve*	*+ve (β-hemolytic)*	*N.R (N.R)*	*N.R (N.R)*	*N.R*	Lactobacillus *sp.*

∗All bacteria were found motile, positive and negative for catalase and Voges proskuer's test, respectively.
[a] = cells/spores dimension in µm, N.R = Not required.

Table 2: Growth optimization of the bacterial isolates in MII medium following 24 hours of incubation.

	p H			TEMPERATURE			Aeration		INOCULUM		
Isolate code.	**5**	**7**	**9**	**Room (25°C±1)**	**37°C**	**50°C**	**Aerated**	**Non-aerated**	**1 %**	**5 %**	**10 %**
Na – A	0.185 ± 0.014	0.112 ± 0.020	0.124 ± 0.002	0.006 ± 0.003	0.195*** ±0.014	0.216*** ±0.020	-0.052 ±0.004	0.0106 ±0.022	0.04 ± 0.06	0.019 ± 0.013	-0.017 ±0.035
Na – B	0.183 ± 0.012	0.158 ± 0.002	0.18 ± 0.007	0.0198 ±0.011	0.177* ±0.006	0.198* ±0.020	-0.064 ±0.002	-0.004 ±0.036	-0.05 ±0.014	0.0376 ±0.017	0.043 ±0.010
Na – C	0.175 ±0.016	0.08 ±0.020	0.179 ±0.005	0.044 ±0.021	0.1774* ±0.057	0.0227Δ ±0.005	0.1464 ±0.015	-0.1013 ±0.003	0.268 ±0.002	0.223 ±0.039	0.269 ±0.008
Na – D	0.382 ±0.021	0.402 ±0.029	0.465 ±0.003	0.083 ±0.030	0.3614 ±0.017	0.055 ±0.008	0.544 ±0.012	0.368 ±0.030	0.622 ±0.005	0.636 ±0.056	0.696 ±0.010

Values represent O.D. of the bacterial cultures and are means of three replicates ± S.E.M . Those with asrerisk (s) are significantly different from the values in the first column of a respective experiments. Difference between the values of 2nd and 3rd columns is indicated by triangle. Single factor of analysis of variance and Student's t-test. ∗, Δ=$p \leq 0.05$; ∗∗∗=$p \leq 0.001$.

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