Research Article

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ANTIBIOTIC SUSCEPTIBILITY OF BIODEGRADING BACTERIAL ISOLATES FROM DAIRY EFFLUENT

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Abstract: The present study investigates the prevalence of antibiotic resistance among bacterial isolates from dairy effluent sample of two different seasons from Verka Milk Plant, Mohali. Twenty bacteria were isolated by spread plating and streaking method. Variable amount of reduction in BOD and COD was observed in dairy effluent after the treatment with free and immobilized seven bacterial strains i.e. A_3, A_8, A_{11}, A_{13}, A_{19}, A_{21} and A_{23}. Bacterial isolates with degrading efficiency were identified on the basis of standard cultural, morphological and biochemical characteristics. Maximum biodegradation was shown by A_{23}. To get ensure about the bacteria used for biodegradation is harmless to the environment these bacteria were evaluated for their resistance and susceptible pattern against ten commonly prescribed clinically significant antibiotics viz, Tetracycline, Cefotaxime, Amikacin, Cefixime, Ampicillin, Penicillin, Gentamicin, Cloxacillin, Erythromycin and Choramphenicol by using antibiotic disc diffusion method. All the strains showed susceptibility against Chloramphenicol, Tetracycline and Cefotaxime. All bacterial isolates has shown resistance against Cloxacillin. The analysis of antibiotic resistance frequencies has shown an incidence of 57.14% strains resistant to four or more than four different antibiotics. The high prevalence of antibiotic-resistant bacteria harboring diverse resistance trait could represent a potential health risk. The study of antibiotic resistance helps to predict future emergence and guide the development of strategies to counteract this resistance before their application in biodegradation process. The present study investigated the prevalence of antibiotic resistance among biodegrading bacterial isolates from dairy effluent and determined their resistance patterns.

Keywords: Antibiotic Disk Diffusion, Antibiotic Susceptibility, Biodegradation, Dairy Effluent, COD, BOD.

INTRODUCTION

Dairy is one of the industries producing wastewater rich in organic matter and thus leading to creation of odorous and high COD containing water. Aerobic treatment of liquid waste produced from food industries and animals is evolving as one of pretreatment option to reduce chemical oxygen demand and biological oxygen demand [10]. Biological treatment is necessary if organic matter is to be removed from water. Nonetheless, biological treatment offers an economical alternative to physical and chemical treatment methods. It is the most widely used method for removal as well as partial or complete stabilization of biologically degradable substances present in wastewaters. The mechanism underlying biological treatment is the decomposition of finely dispersed matter, colloidal and dissolved substances by metabolism of aerobic microorganisms [9]. The rate of biochemical decomposition of waste depends on the activity of microorganisms [11].

Antibiotic resistance refers to the ability of microorganisms to withstand the bacteriostatic and bactericidal effects of antibiotics. It provides a survival benefit to the invading microorganisms and under such circumstances it is difficult to eliminate the infection caused by these microorganisms [1].

Wastewater treatment plants are not capable for the complete removal of antibiotic resistant bacteria, so the resistant bacteria can be found from effluent of wastewater treatment plants [3]. Antimicrobial resistant strains of bacteria are an increasing threat to animal and human health [5]. Only presences of antibiotics in surface water can develop, transfer and maintain antibiotic resistant bacteria in the environment. Antibiotic resistant ability of bacteria can be transferred between different species [3]. The excessive use of antimicrobials has led to antibiotic resistance and particularly multi resistance, which are important public health concerns since they may cause failure of therapeutic treatment [4]. The resistance development may be due to nonspecific mechanism with gene regulation of plasmids and chromosomes, which may be heritable or transferable due to the presence of a resistance (R-factor) factor [2]. The increasing prevalence of antimicrobial resistant bacterial pathogens has severe implications for the future treatment and prevention of infectious diseases in both animals and humans [5]. In the present work, a biotechnological approach has been utilized to treat the dairy effluent using efficient bacteria having bio-degradative potential for dairy effluent. Biodegradation of dairy effluent was studied in terms of reduction of COD; BOD. Another objective was to

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determine the antibiotic resistance patterns of effluent degrading bacteria which were isolated from dairy effluent.

MATERIAL AND METHODS

Effluent Sample:

For the present study the effluent samples were collected in summer and winter seasons in different months i.e. August'11, November'11, February'12 and May'12 in sterile plastic container from Verka Milk Plant Mohali, India.

Media:

For the isolation of the microorganisms from the effluent Nutrient Agar, MacConkey Agar, Eosin Methylene Blue (EMB) Agar and Czapek-Dox Agar were purchased from SRL (Sisco Research Laboratories) and King's B and OF Basal medium was purchased from Hi-Media. The Nutrient agar medium had the following composition (g/l): agar 15.00, peptone 5.00, sodium chloride 5.00, yeast extract 2.00, beef extract 1.00. MacConkey agar medium contained the following ingredients (g/l): peptic digest of animal tissue 17.00, agar 13.50, lactose 10.00. Sodium chloride 5.00, bile salts 1.50, proteose peptone 3.00, neutral red 0.03, crystal violet 0.001. The Eosin Methylene Blue agar medium had the following composition (g/l): peptone 10.00, agar 13.50, lactose 5.00, sucrose 5.00, dipotassium hydrogen phosphate 2.00, eosin Y 0.40, and methylene blue 0.065. King's Medium B agar medium contained the following ingredients (g/l): proteose peptone no.3 20.00, dipotassium hydrogen phosphate 1.50, magnesium sulphate, $7H_2O$ 1.50, agar 20.00. OF Basal medium contained the following ingredients (g/l): casein enzymic hydrolysate 2.00, sodium chloride 5.00, dipotassium phosphate 0.30, bromo thymol blue 0.08, and agar 2.00.

Isolation of the microorganism and characterization of the isolated strain:

0.1ml of the dairy effluent was spread on to the solidified Nutrient Agar medium, EMB, MacConkey, King's B medium and incubated at 37°C for 48 hours. Based on the morphological and biochemical test isolates were identified as *Pseudomonas sp.* (A_3), *Bacillus* sp.(A_8), *Bacillus sp.*(A_{11}), *Staphylococcus sp.*(A_{13}), *E. coli* (A_{19}), Micrococcus sp.(A21) and *Staphylococcus* sp.(A_{23}).

Preparation of seed culture (Inoculum):

Cells from bacterial isolates A_3, A_8, A_{11}, A_{13}, A_{19}, A_{21} and A_{23} were inoculated into 50 ml of LB medium and incubated in a rotary shaker at 37°C for 24 hours.

Preparation of free cells:

100ml of LB medium was inoculated with 1 ml of seed culture and incubated at 37°C in a rotary shaker for 24hrs. Fully grown cells harvested by centrifuging at 5000 rpm for 15min. Washings were given to the cell pellet with 50ml of autoclaved distilled water (D.W.) twice. The pellet was re-suspended in 10ml of autoclaved D.W. out of which 2.5ml of suspension was used as free cells and 7.5ml of suspension was mixed with sodium alginate for immobilization.

Preparation of immobilized cells:

Immobilized cells were prepared by sodium alginate and calcium chloride entrapment method [6].

Biodegradation using free and immobilized cells in shaker flasks:

Degradation of dairy wastewater was conducted in Erlenmeyer flasks using free and immobilized bacterial cells [7].

Estimation of Biological oxygen demand (BOD) and chemical oxygen demand (COD):

The BOD and COD of the samples was determined using titration method [8].

Determination of Antibiotic resistance:

The antibiotic resistance was done by standard agar disc diffusion method on BHIA using commercial discs (Hi-Media, Mumbai, India [2]. 100µl of fresh bacterial cultures were spread on Nutrient Agar plates. The following antibiotics such as Tetracycline (30µg/ml), Cefotaxime (30µg/ml), Amikacin (30µg/ml), Cefixime (5µg/ml), Ampicillin (25µg/ml), Penicillin-G (10µg/ml), Gentamicin (20µg/ml), Cloxacillin (30µg/ml), Erythromycin (51µg/ml), Chloramphenicol (30µg/ml) discos were placed on the plate. Within 15 min of the application of the discs, the plates were inverted and incubated at 37°C. After 24 h of incubation, the plates were examined, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was then translated into sensitive and resistant categories [13].

RESULTS

Identification of effluent degrading strain from the dairy effluent:

The 20 strains were isolated from dairy effluent by spread plate method and isolated using streaking method. Among all, seven strains (A_3, A_8, A_{11}, A_{13}, A_{19}, A_{21} and A_{23}) had shown biodegrading capacity and were characterized using morphological and biochemical tests (table 1).

Biodegradation using Immobilized and Free Cells in Shake Flasks:

Immobilized cells and free cells were prepared for the biodegradation studies. The variations in the COD and BOD of dairy effluent treated with immobilized and free cells of A_3, A_8, A_{11}, A_{13}, A_{19}, A_{21} and A_{23} was determined. From the analysis of the results (table 2) it

was found A_{23} (*Staphylococcus sp.*) free cells and immobilized beads improved the quality of dairy effluent effectively. Percentage Reduction in COD after 24hrs of incubation was 66.87 % and 56.87 % with immobilized and free A_{23} strain, 46.87% and 53.75% with immobilized and free A_{19} strain and 43.75% and 36.87% for A_{13}. Immobilized A_3, A_8, A_{11} and A_{21} had shown increase percentage reduction in COD as compared to free cells. Minimum COD reduction was shown by A_{21} but there were sharp increase in BOD reduction by immobilsed A_{21} strain.

Table.1: Morphological and Biochemical Characterization for Identification of Bacterial Strain

Morphological / Biochemical Characteristics	A_3	A_8	A_{11}	A_{13}	A_{19}	A_{21}	A_{23}
Configuration	Circular	Circular	Echinulate	Circular	Circular	Circular	Circular
Elevation	Raised	Flat	Flat	Raised	Raised	Raised	Raised
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Shape	Rod	Cocci	Cocci	Cocci	Rod	Cocci	Cocci
Gram staining	-	+	+	+	-	+	+
Catalase	+	+	+	+	+	+	+
Citrate	+	+	+	+	-	+	-
MR	-	-	-	+	+	-	+
VP	-	+	-	+	-	-	+

Table.2: Percentage (%) reduction in COD and BOD of the effluent after treatment with free cells and immobilized cells.

% reduce--tion	A3	A8	A11	A13	A19	A21	A23
COD mg /l Free cell	21.1	26.6	22.4	43.7	53.7	20.3	56.9
COD mg /l Immobili-zed cell	39.9	39.2	28.3	36.9	46.9	22.4	66.9
BOD mg /l Free cell	18.1	48.2	16.0	39.2	36.9	16.6	69.7
BOD mg /l Immobili-zed cell	40.5	50.8	58.3	42.1	39.2	47.0	67.2

In the present study, Antibiotic susceptibility test was performed to find out efficient susceptible strain out of seven biodegrading strain that can be used for biodegradation without harming environment. Environments containing antibiotic residues exert selection pressure and contribute to the appearance of resistant bacteria. In the light of the potential health risk, many studies have focused on antibiotic-resistant bacteria from various ecosystems [14].

Susceptibilities of 7 strains were determined for ten antimicrobial drugs using the disc diffusion assay (Tables 3 (a). All isolates were resistant to one or more antimicrobial agents (table 3 (b)). Results are expressed as sensitive (S) (≥12mm), resistant (R) (≤12mm)[12]. Antibiotic susceptibility and resistance patterns of bacterial isolates were shown in fig.1. Cloxacillin was found to be most vulnerable antibiotic against which all bacterial isolates shows 100% resistance. Erythromycin and Penicillin were found another in danger antibiotics as total 4 isolates (i.e. 57.14%) were found to have resistance against theses antibiotics. It has been observed that Amikamicin and Gentamicin were moderate antibiotics as one isolate (14.28%) has shown resistance against it. It has been also observed that all isolates were highly susceptible against Tetracycline, Cefotaxime and Chloramphenicol.

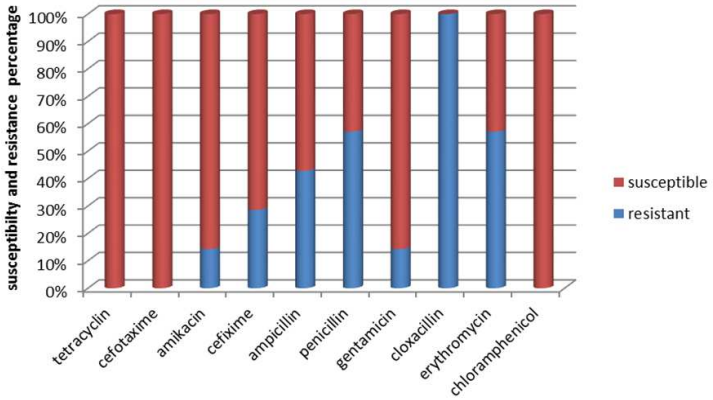

Figure.1: Antibiotic Susceptibility and Resistance Patterns of Bacterial Isolates

Table.3 (a): The Antibiotic Susceptibility Testing of the Bacterial Isolates

Antibiotics (µgm/disc)	Zone of inhibition against antibiotics (mm)						
	A_3	A_8	A_{11}	A_{13}	A_{19}	A_{21}	A_{23}
TETRA-CYCLINE TE30	22	22	23	23	19	26	25
CEFOTAXIME CTX (30)	16	21	27	24	20	24	20
AMIKACIN AK (30)	25	19	21	22	10	12	20
CEFIXIME CFM (5)	-	18	26	22	-	21	16
AMPICILLIN AMP (25)	10	10	17	19	12	12	9
PENICILLIN-G P (10)	10	-	14	17	9	14	-
GENTAMICIN HLG (20)	30	16	13	17	11	12	12
CLOXACILLIN COX (30)	-	8	-	-	-	-	-
ERYTHRO-MYCIN E (51)	30	11	13	10	12	9	9
CHLORAM-PHENICOL C (30)	30	24	28	26	25	29	27

Zone of inhibition including Antibiotic disc diameter i.e.6mm

Table.3 (b): Antibiotic Susceptibility and Resistance pattern of Efficient Bacterial Strains

Antibiotics	Resistance and Susceptibility patterns shown by bacterial isolates						
	A_3	A_8	A_{11}	A_{13}	A_{19}	A_{21}	A_{23}
TETRACYCLINE TE30	S	S	S	S	S	S	S
CEFOTAXIME CTX30	S	S	S	S	S	S	S
AMIKACIN AK30	S	S	S	S	R	S	S
CEFIXIME CFM5	R	S	S	S	R	S	S
AMPICILLIN AMP25	R	R	S	S	S	S	R
PENICILLIN-G P10	R	R	S	S	R	S	R
GENTAMICIN HLG20	S	S	S	S	R	S	S
CLOXACILLIN COX30	R	R	R	R	R	R	R
ERYTHROMYCIN E51	S	R	S	R	S	R	R
CHLORAM-PHENICOL C30	S	S	S	S	S	S	S

The gram positive and gram negative bacterial isolates showed totally different resistance patterns for Cefixime. A_{23} strain had shown high biodegrading capacity but shown 40% resistance against antibiotics whereas A_{11}, A_{13} and A_{21} had shown lower biodegrading capacity as compare to A_{23} but had shown 20% or lesser than 20% resistance. Although, these antibiotics are commonly prescribed antibiotics, increased and uncontrolled use of these antibiotics led to the generation of multi-drug resistant strains [12]. The spread of multiple antibiotic resistant bacteria has been the most serious threat to the successful treatment of disease[2]. The bacteria that showed more resistance for antibiotics as compared to other bacterial isolates is not suitable for use in biodegradation process because it can harm the surrounding and can't cure easily by the mentioned antibiotics treatment. The results provided one of safe assurance of using these strain to in situ bioremediation of dairy effluent.

CONCLUSION

Microbial treatment of dairy effluent is a good approach to treat effluent with much less expenses but before using any bacteria, antibiotic resistance pattern of efficient strain should be checked out for our safety concern. Like in this present study A_{23} strains showed highest biodegrading capacity among all the strains but due to its resistance against four antibiotics it cannot used in biodegradation as a first preference. Rather A_{11}, A_{13} and A_{21} can be used in biodegradation without any hesitation because of their low resistance pattern. The high prevalence of antibiotic-resistant bacteria harboring diverse resistance trait could represent a potential health risk. The present study evaluated the prevalence of antibiotic resistance among biodegrading bacterial isolates from dairy effluent and determined their resistance patterns. The study of antibiotic resistance helps to predict future emergence and guide the development of strategies to counteract this resistance before their application in biodegradation process.

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