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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF BACTERIA FROM COOLANT OILS

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ABSTRACT: Coolants are hydrocarbons, used to lubricate parts of machines for smooth performance. While in use, a coolant quickly gets contaminated with foreign materials, making it less effective and unpleasant odors are developed due to microbial action. Hence coolants need to be replaced frequently. The expense of disposing used coolants and replacing it with fresh coolants adds significantly to the manufacturing cost. The present study is focused on isolation, identification and characterization of coolant oil contaminating bacteria as an initial step to solve these contamination problems. Used and unused samples of coolant were collected from oil stations, auto mechanic workshops and steel industry for the isolation of the contaminants. Ten dominant bacterial isolates of the genus *Pseudomonas, Staphylococcus, Micrococcus, Salmonella, Cellobiococcus* and *Pneumonia* were identified by morphology, biochemical tests and PIB tool. Isolates were subjected to four different media, various pH and temperatures for characterization of optimal conditions of growth. *Pseudomonas pseudomallei, Micrococcus luteus 3, Micrococcus varians* and *Salmonella ferlac* were observed in mineral, synthetic and aerobic media, *Staphylococcus hyicus*, *Cellobiococcus species* and *Staphylococcus intermedius* in synthetic and aerobic fermentation media and *Pseudomonas cepacia, Pseudomonas piketti* in mineral salt and aerobic fermentation media. The ten isolates showed optimal growth at different temperatures between 20°C and 90°C and different pH, ranging from acidic to alkaline. In conclusion, the used coolants harbor hazardous pathogens such as *Pseudomonas* species which multiply rapidly and survive high temperatures. These isolates could be targeted for further studies on development of antidotes as a solution to the coolant contamination problems.

Key words: Coolant oil, Biochemical tests, Oil degrading bacteria, PIB tool, Bacterial identification

Abbreviations: I*: Industrial used sample; B*: Bike used sample; C*: car used sample; I: Industrial unused sample; B: Bike unused sample; C: car unused sample PIB: Probabilistic Identification for Bacteria

INTRODUCTION

Engine oil is a complex mixture of hydrocarbons and other organic compounds including some organometallic constituents that are used to lubricate parts of automobile engines for smooth function[1, 2]. Hence these are also called as coolants. They are cutting fluids that are used in machines to cool and lubricate the tool. In addition to the cooling effect, the oil also saves the engine by sealing, cleaning and inhibiting corrosion[1-4]. Coolant oil concentrates are practically free of bacteria when sold to the customer. The open air circulation systems offer ample opportunity for the emulsion to become contaminated with bacteria as it flows over the hands of workers and collects in sumps / individual tanks around the machine[4-7].

The used coolant collected in the sump forms a layer of tramp oil on the surface depleting oxygen, creating an ideal environment for anaerobic bacteria to thrive. And it is these bacteria that degrade coolants[7]. The bacteria use nutrients containing carbon, hydrogen, nitrogen, sulfur and traces of phosphorous, magnesium, calcium and other components of the coolants[8-10]. Due to this microbial activity the oil contamination increases over time. The acidic byproducts of the microbes reduce the pH of coolants which becomes corrosive to work pieces and also cause rancidity of the oil[11-14]. The coolants used in metal processing are highly contaminated with potentially pathogenic bacteria which may be associated with the respiratory tract and other infections in exposed workers. Contaminated coolants can be used only for a short time. The disposal of this contaminated coolant is usually in the nearby water bodies or on the soil surface which leads to environmental pollution[15-18]. Proper coolant maintenance by routine removal of tramp oil, metal chips, cleaning the sump, adding biocides and also maintaining the proper coolant–water mix and additives in beneficial concentration may prevent bacterial growth[19, 20]. It was observed that bacteria flourished in the coolants even after using the different preventive measures [21-26]. The present study was focused on the isolation, characterization and identification of oil degrading bacteria as a first step to solve the coolant contamination problems in open systems.

MATERIALS AND METHODS

Growth and maintenance of bacterial cultures

Nutrient broth was used for growth and characterization of the bacterial strains. For identification, selective media and different biochemical methods were used. Stock bacterial cultures maintained by periodic sub culturing on the nutrient agar plates were stored at 4°C.

Collection of sample

Different commercially used and unused samples of coolants from steel industry, used and unused samples from two and four wheeler vehicles were collected from auto mechanic workshops, in sterile vials and stored for further use.

Sample preparation

50 ml of the used and unused samples were subjected to different temperatures such as 20°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C for 15-20 minutes, centrifuged at 3,500 revolutions per minute (rpm) for 30 minutes. Sediments were dissolved using 1 ml sterilized distilled water and stored for further study[9].

Isolation of bacterial strains

Nutrient agar plates were prepared. Sterility test was carried out by keeping the plates overnight. A sterile micropipette tip was used to dispense 50 µL from each dissolved sediment samples on to nutrient agar plates. Glass spreader, dipped in alcohol, flamed and cooled was used for spreading on the plates. The plates were incubated at 37°C for 18 hours along with a control. The plates were observed for bacterial colonies[9, 12].

Screening of micro-organisms

From the bacterial colonies, which appeared on the nutrient agar plates, 27 bacterial isolates were picked randomly, purified and streaked on nutrient agar plates, The plates were incubated at 37°C for 18 hours. The isolates that showed the best growth on these nutrient plates were used for further study[12, 27].

Characterization of selected isolates

Identification and characterization of selected pure bacterial isolates were performed by morphological, biochemical tests such as shape, arrangement, colonies, temperature, growth, form, margin, elevation, density, clouding, indole production test, methyl red and Voges- Proskauer test, citrate utilization, starch hydrolysis, urease test, hydrogen sulfide production, catalase test, tween 80 test, NaCl test, Christensen's citrate agar test, Mac Conkey's test, growth at room temperature, citramide test, gelatin hydrolysis, lactose, glucose, sucrose fermentation tests and effect of metals according to Faddin and Bryant[27, 28]. Results obtained on the basis of visual change in color produced by bacterial isolates were used for further identification.

Identification of bacterial isolates using Probabilistic Identification for Bacteria Windows (PIB Win) Current Version 2.0

The PIB win program provides pro identification of unknown bacterial isolates against identification matrices of known strain[29]. The program makes use of excel (2007) files to score identification matrices and results display.

Steps followed:

- The website 'Identification of gram positive/gram negative bacteria by T.N. Bryant' was selected
- A web page was displayed with all different types of experiments.
- According to the results obtained, left click was done on the positive or negative boxes given at the side of each experiment.
- Submit button was clicked at the bottom of the window.
- The identification results - table with the name of the organism and its score was displayed.
- The above procedure was repeated for each of the organisms.
- The results were then captured by using screen capture software.

The results were confirmed on the basis of the highest ID score of each organism.

Survival of isolates in different media

The different fermentation media -mineral salt (media I), synthetic (media II), aerobic (media III) and anaerobic (media IV) were prepared and the identified 10 isolates were inoculated under sterilized conditions incubated at 37°C for 18 hours and the results recorded[28].

Survival of isolates at different temperatures

The identified isolates were inoculated aseptically on to the labeled nutrient agar plates and incubated at different temperatures i.e., 20°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C for 18 hours[30]. The plates were observed for the colony growth at different temperatures and the results recorded.

Survival of isolates at different pH

The broth was prepared using different buffers of pH 2.2, 4, 6, 8 and 10 were prepared according to the procedure mentioned in materials and methods [30]. The inoculated test tubes were incubated at 37°C for 18 hours and the growth of organisms was measured by taking optical density (OD) at 540 nm.

RESULTS

Isolation and selection of coolant contaminated bacteria

The number of bacterial colonies developed on nutrient agar plates for used and unused samples were different (Table 1). However, no colonies were observed in industrial unused sample.

Table 1: Number of morphologically different colonies isolated from cultured, used and unused coolant samples from Industry, two wheeler and four wheeler vehicles.

sample	No of colonies
I*	05
B*	07
C*	06
I	Nil
B	05
C	04

I: Industrial used sample; B*: Bike used sample; C*: car used sample; I: Industrial unused; B: Bike unused; C: Car unused. Values are mean of number of colonies from three plates*

Pure colonies of the isolates showing the same characteristics were omitted and ten isolates showing different characteristics (Table 2) were named based on whether samples is unused (I/ B/ C) or used (I*/ B*/ C*) followed by the incubation temperature (20°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C) at which the colonies appear as I*20, I*60, I*70, I*90, B70, B80, B*60, B*70, B*80 and C*60. As given by the PIB Windows Current Version 2.0, *Staphylococuss hyicus, Pseudomonas pseudomalli, Pseudomonas cepacia, Pseudomonas piketti, Micrococuss leutus, Salmonella ferlac, Cellobiococcus species, KIebsiella pneumoniae aerogens, Staphylococcus intermedius,* and *Micrococcus varians* were identified (Table 3).

Table 2: Biochemical characterization of bacterial isolates

Isolate No	In	Mr	Vp	H_2S	Cit	St.H	GF A	GF G	LF A	LF G	MF A	MF G	SF A	SF G	UH	NaCl	T 80	Cat	CCA	Gr-T	Mac	G H	Gram stain
I+ 20	-	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Cocci+
I+ 60	-	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Cocci+
I+ 70	-	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Rod-
I+ 90	-	+	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Rod-
B*60	-	+	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Cocci+
B*70	-	-	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	Rod-
B*80	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	Cocci+
C+ 60	-	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	Cocci+
B70	-	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	+	-	+	Rod+
B80	-	-	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	Rod-

***In** Indole, **Mr** Methyl red,**VP** Voges- Proskauer test , **H_2S** Hydrogen sulfide test, **Cit** Citrate, **St.H** Starch hydrolysis, **GF** Glucose fermentation, **LF** Lactose fermentation, **MF** Maltose fermentation, **SF** Sucrose fermentation, **UH** Urea hydrolysis, **NaCl** Growth in presence of 1% Nacl,**T 80** Tween 80 test **cat** Catalase **CCA** Christiens citrate agar, **Gr-T** Growth at room temperature, **Mac** Mackonkeys broth, **GH** Gelatin Hydrolysis. Cocci+ Gram Positive Cocci, Rod- Gram negative Rod, + positive test, - negative test. A- acid production, G- gas production*

Table 3: Identification of the isolates using Probabilistic Identification for Bacteria (PIB) Windows Version 2.0 Tool

Name of the Isolates	ID SCORE
Staphylococcus hyicus	0.64801
Staphylococcus intermedius	0.42768
Micrococcus varians	0.90427
Micrococcus luteus 3	0.60164
Pseudomonas pseudomallei	0.80411
Pseudomonas cepacia	0.56258
Pseudomonas pikette	0.66154
Salmonella ferlac	0.45820
Klebsiella pneumoniae .aerogenes	0.90603
Cellobiococcus sp	0.45518

In different fermentation media, isolates showed different level of growth (Table 4). None of the isolates thrived in anaerobic agar media (media III), whereas growth of all isolates was observed in aerobic agar media (media IV). Except *Pseudomonas cepacia* all isolates showed the growth in synthetic media (media II). In mineral salt media (media I) three *Pseudomonas species,* two *Micrococcus species, Salmonella ferlac* and *Klebsiella pneumoniae* showed growth while *Staphylococcus hyicus*, *Cellobiosococcus species* and *Staphylococcus intermedius* did not grow. Isolates when incubated at different temperatures i.e., 20˚C, 40˚C, 50˚C, 60˚C, 70˚C, 80˚C and 90˚C showed that, *Staphylococcus hyicus grows* over a very wide range of temperatures varying from 20˚C, 40˚C, 50˚C, 60˚C, 70˚C, 80˚C and 90˚C while *Pseudomonas pseudomallei* varies at a lessor wide range 50˚C, 60˚C, 70˚C, 80˚C and 90˚C. Some other isolates grew over a even narrower temperature range i.e., *Pseudomonas cepacia, Cellobiosococcus species at* 60˚C and 70˚C and *Pseudomonas piketti* at 80˚C and 90˚C. The remaining isolates grew only at a single temperature - *Micrococcus luteus 3,* and *Klebsiella pneumoniae* at 70˚C, *Staphylococcus intermedius* and *Salmonella ferlac* at 80˚C, and *Staphylococcus varians* at 60˚C (Table 5).

Table 4: Growth of bacterial isolates on different fermentation media

Name of the Isolates	Fermentation Media			
	Mineral salt (Media I)	Synthetic (Media II)	Aerobic (Media III)	Anaerobic (Media IV)
Staphylococcus hyicus	-	+	+	-
Staphylococcus intermedius	-	+	+	-
Micrococcus varians	+	+	+	-
Micrococcus luteus 3	+	+	+	-
Pseudomonas pseudomallei	+	+	+	-
Pseudomonas cepacia	+	-	+	-
Pseudomonas pikette	+	+	+	-
Salmonella ferlac	+	+	+	-
Klebsiella pnuemonia aerogens	+	+	+	-
Cellobiococcus species	-	+	+	-

+ Growth observed - No growth observed

5: Survival of bacterial isolates at different temperatures

Organisms	Temperature in °C						
	20	40	50	60	70	80	90
Staphylococcus hyicus	+	+	+	+	+	+	+
Staphylococcus intermedius	-	-	-	-	-	+	-
Micrococcus varians	-	-	-	+	-	-	-
Micrococcus leuteus	-	-	-	-	+	-	-
Pseudomonas Pseudomalli	-	-	+	+	+	+	+
Pseudomonas cepacei	-	-	-	+	+	-	-
Pseudomonas piketti	-	-	-	-	-	+	+
Salmonella farlac	-	-	-	-	-	+	-
KI pneumonia aerogens	-	-	-	-	+	-	-
Cellobiococcus species	-	-	-	+	+	-	-

+ Growth observed *- No growth observed*

Isolates grown in different pH environment have shown that *Staphylococcus hyicus, Cellobiosococcus species, Staphylococcus intermedius,* and *Pseudomonas cepacia* can thrive at acidic pH (pH 4.0). *Klebsiella pneumoniae* preferred alkaline pH (pH 8.0), while *Micrococcus luteus 3*, *Salmonella ferlac, Pseudomonas pseudomallei, Micrococcus varian* and *Pseudomonas piketti* showed substantial growth at a less acidic pH of 6.0 (Table 6). The pH of the used coolants were acidic as compared to the unused coolants which were slightly alkaline in nature (Table 7).

Table 6: Survival of bacterial isolates at different pH

Organisms	Optimum pH
Staphylococcus hyicus	4
Staphylococcus intermedius	4
Micrococcus varians	6
Micrococcus leuteus	6
Pseudomonas pseudomalli	6
Pseudomonas cepacei	4
Pseudomonas piketti	6
Salmonella farlac	6
Klebsiella pneumoniae aerogens	8
Cellobiococcus sps	4

I*: Industrial used sample; B*: Bike used sample; C*: car used sample; I: Industrial unused; B: Bike unused; C: Car unused

Table 7: pH of Used and unused samples

Sample	pH
I*	5.5
B*	5.8
C*	5.2
I	7.9
B	7.7
C	7.8

DISCUSSION

Coolants / cutting fluids improve productivity and extend tool life by cooling and lubricating the cutting tool. However, while performing these functions coolant quickly becomes contaminated with foreign materials resulting in loss of effectiveness and development of foul odor [8, 13, 14, 18]. As a result of which coolants have to be replaced often. As a result, in recent years, the bacterial content of cutting fluids has received considerable attention[7]. Research has been directed towards understanding the physical and chemical properties of cutting fluid that makes it susceptible for microbial degradation[11-14], determination of the disease producing organisms nourished by the emulsion oils under industrial conditions which are the root cause of contamination and the mechanisms by which these bacteria degrade the fluid[7, 16, 17].

One way to reduce both environmental pollution and the disposal cost of a cutting fluid is to prolong its life, which opens the way to investigate the degradation dynamics of the cutting fluid system[11]. However, proper coolant maintenance may prevent bacterial growth only to some extent[13]. Coolant maintenance incorporates maintenance of the proper coolant to water ratio in the mix with appropriate additive concentration[13], or inclusion of other additives such as detergents/dispersants (which keep the engine clean by minimizing sludge build up)/corrosion inhibitors or alkaline additives (neutralize acidic oxidation products of the oil). Some commercial cutting oil inhibitors may even contain substances which favor bacterial growth[22].

Till date, a lot of attention has been centered around the use of bacteria in bioremediation of oil to combat the environmental hazards like oil spillages[1, 11, 31]. Yet, no review has been reported on isolation of bacteria from used coolant oils even though it is one of the most important and expensive causes of degradation[1, 2]. This study attempts to isolate characterize and identify coolant degrading bacteria from different commercial coolants used in automobiles (two wheeler, four wheeler) and also the steel industry.

Cutting fluid supports aerobic, anaerobic microorganisms including bacteria and fungi[7, 11]. The acidic byproducts of the microbes reduce the pH of the cutting fluid, which is corrosive for most types of machine work pieces as well as the machine tool[7, 11]. Among the bacterial isolates isolated in this study, the most common potentially pathogenic genus cultured from used cutting fluid are *Pseudomonas species* and *Klebsiella pnuemoniae* which are known to degrade oil[4, 7, 13]. Furthermore, *Pseudomonas species* has been noted as one of the major bacteria that help degrade most organic pollutants as they possess plasmids carrying catabolic genes[32]. Eight of the ten bacterial isolates of this study namely *Staphylococuss hycus, Pseudomonas pseudomalli, Pseudomonas cepacia, Pseudomonas piketti, Cellobiococcus species, KIebsiella pneumoniae, Staphylococcuss intermedius,* and *Micrococcus varians* were from used coolants and two isolates *Micrococuss leutus, Salmonella ferlac* from unused coolant (Table 2). *Pseudomonas* and *Acinetobacter* species are the most common bacterial hydrocarbon degraders reported in literature [2, 3, 17, 33-37].

Three classes of bacteria typically encountered in petroleum products include (i) Sulfate reducing bacteria (SRB) which metabolize sulfate (ii) Acid producing bacteria (APB) which ferment sugars to acids (typically highest organic acids) and (iii) General aerobic bacteria (GAB)[38]. All isolates of this study were found to be SRB (Table 2), APB (Table 7), and also aerobic in nature as no growth was observed in anaerobic media (Media III) (Table 6) which correlates with an earlier report[38]. SRB species are the main reason for the rancidity/stale odor observed in contaminated coolants[14], and ARB reduces the pH of the cutting fluid making it corrosive for most types of work pieces including the machine tools[14]. However, studies on, the combined roles of aerobes and SRB in deterioration of coolant oil indicates a two phase spoilage process - the first phase involving aerobic bacteria and the second phase involving anaerobic SRB[8, 11], resulting in acidic pH of the coolant which correlates with our findings (i) isolates are all aerobic (Table 4) (ii) fresh coolants which are slightly alkaline to start with, become acidic with use (Table 7).

Reports on optimum temperature characterization of contaminating microbes have shown highest degradation of coolants at temperatures as low as 35°C by *Bacillus mycoids*[1], as well as tolerance to elevated temperatures by *Pseudomonas species* isolated from hydrocarbon contaminated areas[30]. Characterization of the isolated ten microorganisms of this study has shown varying tolerance to temperature ranging from as low as 20°C to as high as 90°C (Table 5). *Staphylococcus hyicus* and *Pseudomonas pseudomallei* shows tolerance over wide range of temperatures while the other isolates flourished either at a narrower range (*Pseudomonas cepacia, Cellobiosococcus species* and *Pseudomonas piketti*) or at single temperatures (*Micrococcus luteus 3, Klebsiella pneumoniae*, *Staphylococcus intermedius, Salmonella ferlac* and *Staphylococcus varians*). Since the cutting fluid is exposed to varying temperatures and air, in the open coolant system of industry, the condition favors the growth of the aerobic bacteria resulting in contamination[5, 14].

Aerogenous infections have become increasingly frequent among workers in all industrial branches, particularly in metal processing. A study undertaken has shown a higher incidence of respiratory system diseases, including chronic cough, increased sputum secretion, functional disorders, pharyngitis, laryngitis, sinusitis and rhinitis in exposed workers compared to non exposed workers which has been related to pathogenic bacteria in the working environment[13,24,39]. Although it has been reported that pathogenic bacteria such as S*treptococcus species* and *Micrococci* are not capable of surviving in cutting oils[36], this study is in partial agreement showing absence of any *Streptococcus species,* but presence of two species *of Micrococcus i.e., Micrococcus leutus,* and *Micrococcus varians* in used coolant samples. Another pathogenic bacterium, *Klebsiella pneumonia aerogens,* was isolated from used coolant oils in this study as well. *Klebsiella pneumoniae* was earlier isolated from 32 of the 100 coolant samples studied in an earlier report[9].

Earlier reports on bacterial inhibitors for cutting oil reveal that commercial cutting oil inhibitors were unable to inhibit all bacteria for significant period of time[22,24]. Pasteurization of the emulsion oil is also not a practical solution[7]. It becomes obvious that there is a need for alternate bacterial inhibitors to save the coolants.

In conclusion, of the ten bacterial isolates obtained in this study from the coolants, three belong to *Pseudomonas* and two belong to the *Micrococcus* genus both of which are potential pathogens and hazardous to the health of the workers. Characterization has revealed them to be SRB, APB and aerobic in nature. Considering the need for an effective control on the microbial growth, these isolates could be explored as potential target organisms to determine the efficacy of inhibitors against them, thus increasing coolant life and decreasing health hazards to the industry workers.

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