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Chemical Control of Aerobic Bacteria Causing Biodegradation of Oil Emulsion

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SUMMARY
This research includes the study of growth and control of aerobic bacteria in oil emulsions , samples of contaminated emulsions were collected from different industrial plants while emulsions were in case of circulation (during work) , Thirty four bacterial isolates were isolated and diagnosed depending on their morphological and biochemical characteristics , more than half of them (20/34) were primary invaders and showed the ability to consume the components of oil emulsion (That's are the base oil 40 and the chemical additive known as the concentrate) as a sole source of carbon and energy but the remaining fourteen isolates failed to do so and were considered as secondary invaders , the effect of adding water-soluble chemical compounds including EGME , chlorhexidine gluconate, Na-Tetraborate, Tween-80 as biocides at different concentrations were also studied, The results showed that three of these compounds had a significant effect on controlling biodegradation of the emulsion and their lethal effect increased with increasing concentration .
KEYWORDS; *Biodegradation , aerobic bacteria , biocides , oil emulsion , hydrocarbons , biodeterioration , MWFs .*

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I. INTRODUCTION

large quantities of Metal working fluids (MWFs) are used in mechanical and metallurgical industries to facilitate various operations such as cutting , grinding, drilling as well as for lubricating , cooling and corrosion prevention , as functions of these fluids are different, their composition will becomplexand different as well , MWFs usually composed of a mixture of various hydrocarbon compounds with emulsifiers such as petroleum sulphonate , fatty acid esters , soaps and water, these components creating a nutritional support required for growth of a wide range of microorganisms (1,2,3) also, the open type work operations and the prolong period of use of emulsions leads to severe contamination by microorganisms causing their deterioration , resulting in formation of viscous substances , unpleasant odors and changes in their standard specifications unless the use of appropriate biocides to control microbial growth and in most cases, the compounds that liberate formaldehyde or phenolic derivatives are often used , but since the toxicity of these compounds is not optional, so the concentrations required to control growth and those limits dangerous to health are close to some , and It is not excluded that workers will exhibit allergic skin injuries, especially on hands, due to added biocides (4) . During the past decades, bacterial resistance to new antibiotics used in medical fields has been recorded . Recently, the same problem was observed with the biocides used in industry , sometimes, this resistance is to more than one biocide (5). In a previous study, Saha&Donofrio(6) explained that the sustained growth of high concentrations of cells (10^8 cell / ml) of *P. pseudoalcaligenes* in a main tank of a factory is not affected by the calculated amount of biocide, and members of this genus have the ability to break down many of compounds used as biocides and biofilm layer requires continuously addition of larger quantities of biocides to replace the quantities consumed . The biodegradation of oil emulsions will have a clear negative economic impact due to the shortening of oils lifespan , in addition to other health and environmental problems, and because of the limited researches related to controlling biodegradation of oil emulsions, especially locally produced, despite their extensive use and the existence of the problem in many of our industrial facilities and the inefficiency of imported chemical additives , this research was done .

II. MATERIALS AND METHODS

1-Samples Collection

Samples of used oil emulsions (after 8 working hours only) were collected in sterile 50 ml bottles from different industrial plants . Samples were kept in refrigerated containers and transferred to the laboratory directly . Standard oil emulsion sample and its constituents (the base oil 40 and the concentrated chemical additive known as the concentrate) were obtained from Al-Wasat Refineries / Baghdad.

2 - Bushnell & Haas liquid mineral salts medium had been used to isolate bacterial species which have the ability to utilize hydrocarbons . The isolates were diagnosed and identified using standard methods and diagnostic procedures (7,8,9) . Isolates showed the ability to utilize oil emulsion as a sole source of carbon and energy were chosen to study their ability to utilize the basic components of the emulsion represented by the base oil 40 and the concentrated chemical additive according to the method indicated by Budziniski*et al*(10) .

3 - Total viable cell counts

The replica plate dilution method was adopted (7) .

4 - Preparation of bacterial consortium

The method mentioned by (10,11) in preparing bacterial inoculum for the eight isolates was followed.

5 - Use of biocides

Four types of chemical compounds were used as biocides against bacteria grown in liquid mineral salts medium with 4% oil emulsion , different concentrations of each biocides were used as below

A - Chlorhexidine gluconate in concentrations of 0.5, 1, 1.5, 2, 2.5%

B - Na- Tetraborate in concentrations of 1, 2, 3, 4, 5%

C - Tween - 80 in concentrations of 6,8,10,12, 14%

D - Ethylene glycol mono ethyl ether in concentrations of 2.5, 3, 3.5, 4 , 4.5%

6 - Experimental conditions

A separate flasks of 500 ml containing 100 ml oil emulsion was inoculated with 0.4 ml of bacterial cell (6.2 x 10^6 cell / ml) for each dilution ofbiocides . Flasks were incubated for 6 days at a temperature of 37 ° C , Numbers of viable cells were calculated every 24 hours by replica - plate dilution method , experiments were carried out with three replicates and the average readings were taken , control samples were examined too (without biocides) , a special design was used in the experiment as indicated by (12) to ensure continuous aeration and circulation that was mimic to working machines in industrial plants .

III. RESULTS

Table (1) shows the number of isolates of primary and secondary invaders and their types, a total of 34 bacterial isolates were isolated from deteriorated oil emulsion samples , 20 isolates (58.8%) belong to eight different genera, all are gram negative , showed ability to grow and utilize oil emulsion or its basic components as a sole source of carbon and energy , thus considered as primary invaders and the study was completed on them, While the remaining 14 isolates (41.1%) failed in that and their numbers decreased in the medium until they disappeared and considered to be secondary invaders .Figure (1) shows the growth of bacterial isolates and their numbers increase in mineral salts medium containing 15% base oil 40 . The isolates showed different growth curves associated with increasing numbers since the first day of incubation , as for *C. koseri* and *k. oxytoca* , their numbers reached at the end of the first day $5.3x10^5$ and $6.2x10^5$ cell / ml respectively, then the numbers began gradually to decline with time , but*S. marceescens* , *A.baumanni* , *P.putida* showed a continuous increase in numbers for the first three days amounting to $4.8x10^5$, $6.5x10^5$ and $7.3x10^5$ cell / ml respectively , the numbers were relatively stable for the latter species while the first two showed fluctuation in numbers by continuing incubation days . The isolates of *A.media* and *B.cepacia* showed an initial decrease in numbers for the second and third days followed by continuously increase reaching by the end of the twelveth day $8.6x10^5$ and $8x10^5$ cell / ml respectively, *A.baumanni* and *S.marcescens* recorded the highest numbers up to $10.2x10^5$ and $10.5x10^5$cell / ml at the end of the experiment , While *R.eutropha* showed the lowest numbers increase by the end of the twelfth day and reached $2.8x10^5$cell / ml . Figure (2) illustrates the increase in number of bacterial cells in liquid mineral salts medium containing 10% of the chemical additive known as the concentrates , all isolates showed the ability to grow and utilizing it as a sole source of carbon and energy, however , they all went through a lag phase period on the first day , then numbers began to increase dramatically for subsequent days and reached the highest numbers on the third day for isolates *P.putida* $10.1x10^4$ and *B.cepacia* $9.9x10^4$, while the highest numbers of*S.marcescens* $10.2x10^4$,*C.koseri* $10.1x10^4$ and *A.baumanni* $8.2x10^4$cell / ml were seen on the fourth day, then cell numbers for most isolates began to fluctuate, giving different growth curves for the following days. Figure (3) illustrate the effect of addition of chemical compounds used as biocides to control bacterial growth in 4% oil emulsion compared to control samples. The results were represented by calculating viable cell number for each case.Emulsions containing different concentrations of chemical compounds showed different effects on cell numbers either for each single compound or among different compounds , the addition of chlorhexidine gluconate ,Na-Tetraborate , E.G.M.E showed an increase in controlling bacterial growth with

increasing concentrations . Low concentrations of them (1% , 1.5% of Chlorhexidine gluconate , 2% , 1% of Na-Tetraborate and 2.5% , 3% of E.G.M.E.) had weak effects , However, the effect was evident when increasing concentrations and the lowest concentrations that killed all cells after 24 hours were 2%, 4%, and 4.5% for chlorhexidine gluconate , Na-Tetraborate and E.G.M.E. respectively , While the addition of tween-80 did not show any growth-inhibiting effects but on the contrary, its presence helped an increase in numbers .

IV. DISCUSSION

It is clear from Table 1 that, despite the initial isolation of 14 bacterial isolates belonging to six different genera, three of them are gram negative and three positive, none of them showed the ability to utilize oil emulsion or its basic components as a sole source of carbon and energy, this can be attributed to its loss ofoxidizing enzymes for hydrocarbon compounds, or their ability to consume the easier and more readily available compounds only, so considered as secondary invaders that can grow only in the presence of primary invaders or after the completion of the primary oxidation of hydrocarbons , which enables them to use the metabolic by- products of the first process , or that the growth of primary invaders leads to the consumption of the inhibitory compounds present in the emulsion and production of CO_2 or other products that improve conditions for its growth (13,14) , A number of studies indicated that many bacterial species are capable of growing in oil products as mixed cultures causing or contributing in bio-degradation, despite their inability to utilize the oil derivative if they were grown alone (15,16). The Isolates showed different growth capacities in utilizing base oil 40, and there was a slight decrease in numbers of *B.ceoacia* and *A.media* in the early days of incubation, the reason may be attributed to the need of adaptationperiod and stimulation of enzymatic systems or that the oil contains some compounds affecting cells by inhibiting growth for a period and then lost their effect over time either because of their direct interaction with cells or chemical reactions with metabolites leads to lower their concentrations (17,18) . All isolates showed a lag period at the beginning of incubation with the chemical additive (The concentrate) and this may be due to the same reasons mentioned with the base oil 40, but the rapid subsequent growth indicates that a portion of the components is easily utilized and its bioavailability is high , the chemical additive contains many components such as emulsifiers and inhibitors to prevent rust , corrosion , foam formation , oxidation , color change and production of odors , all can support bacterial growth , However, the important thing is that the chemical additive contains biocides and bacterial ability to grow in it indicates it possesses an effective enzymatic system by means of which it can utilize these substances , this corresponds to what researches stated in that low concentrations of chemical additives that are insufficient to kill or Inhibit cells , may support growth because it constitutes food sources for them (19,20) . In industrial systems, many oxidizing and non-oxidizing biocides are used, but many fail to control microbial growth due to incorrect selection and imprecise use , knowing the type of microorganisms to be disposed of, the dynamics of work , type of operations , microbial ability to form resistant strains and using correct concentrations of biocides are important points in the success of preventing biofouling (21,22) The aim of using biocides in metal working fluids is to make the number of microbial cells at low levels from the beginning or to reduce their numbers when they reach high levels and despite the absence of specific standard controls governing the permissible limits of numbers in such fluids, there is almost a consensus in researches that numbers of aerobic bacteria above 10^5 - 10^7 cell / ml should be avoided (1,5,6) , The calculation of viable cell number over time with the presence or absence of biocide was adopted to show its efficiency and knowing the effect of different concentrations of each biocide , this corresponds to what was mentioned in a number of studies that followed the same principle (10,12,13) .Figures (3) show that concentrations 0,5% of chlorhexidine gluconate , 1% of Na-Tetraborate and(3% & 2.5%) of EGME were not effective in controlling bacterial growth as numbers decreased gradually so the effects of biocides at these concentrations were gradual and weak , then an increase in numbers occurred indicating that the remaining cells had been adapted to biocides and this adaptation may occur due to the action of recessive enzymes that become important in adverse and unsuitable growth conditions , therefore, it is dangerous to use such sub - lethal concentrations as it helps in the emergence of resistant strains that may be highly effective in biofouling (22,23) . The method indicated by (24)in preparing bacterial inoculum was used , Consequently bacterial consortium found in culture medium will be affected by different degrees of biocide due to physiological differences among them , accordingly, the concentrations that showed a high ability to kill all bacterial species in oil emulsions represent the minimum concentrations and this is better than MIC or minimal bactericidal concentration (MBC) calculated for each species separately , as it does not accurately represent the concentrations required to be used in the field of work to prevent growth of all various bacteria present in MWFs , to determine the ability and effectiveness of any biocide to be used , it should be examined against a number of different isolates and the readout rate is taken or MIC 90 is used, which is the lowest concentration that leads to the suppression of 90% of cells , and this what is usually recommended (22) .Tween 80 showed a weak ability to control cell growth at the concentrations used , numbers continued to increase and exceeded in some concentrations, their numbers in the control Figure 3. The reason may be attributed to increasing constituent dissolving of the emulsion making them more emulsified and readily

bioavailable , This corresponds to what others mentioned in that the presence of some hydrocarbon compounds (including Tween 80) affects surface tension , and this speeds up their consumption , although it can prevent or inhibit biodegradation process due to its toxicity (25,26) .

Table 1 ; Distribution of bacterial species isolated from used oil emulsion as being primary or secondary attackers .

Bacterial species	No. of isolates	Percentage %
1- Primary attackers	20	58.82
Pseudomonas putida	4	11.76
Burkholderia cepacia	3	8.82
Acinetobacter baumanni	3	8.82
Serraciamarcescens	2	5.88
Citrobacterkoseri	2	5.88
Aeromonas media	2	5.88
Klebsiella aerogenosa	2	5.88
Ralstoniaeutropha	2	5.88
2- Secondary attackers	14	41.17
Staphylococcus aureus	4	11.67
Escherichia coli	3	8.82
Micrococcus luteus	2	5.88
Corynebacterium amycolatum	2	5.88
Moraxella lacunata	2	5.88
Methylobacteriumorganophilum	1	2.94
Total number	34	99.99

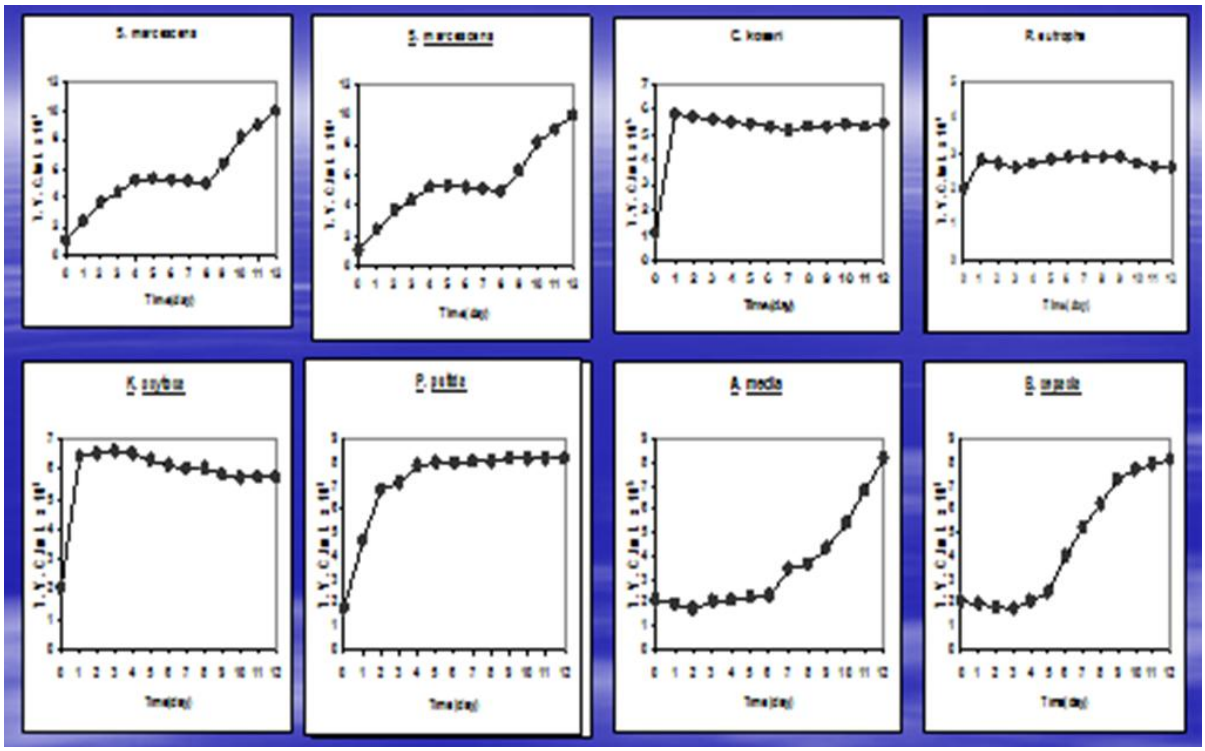

Figure (1) Growth of bacterial isolates in liquid mineral salts medium containing 15% base oil - 40 as a sole source of carbon and energy.

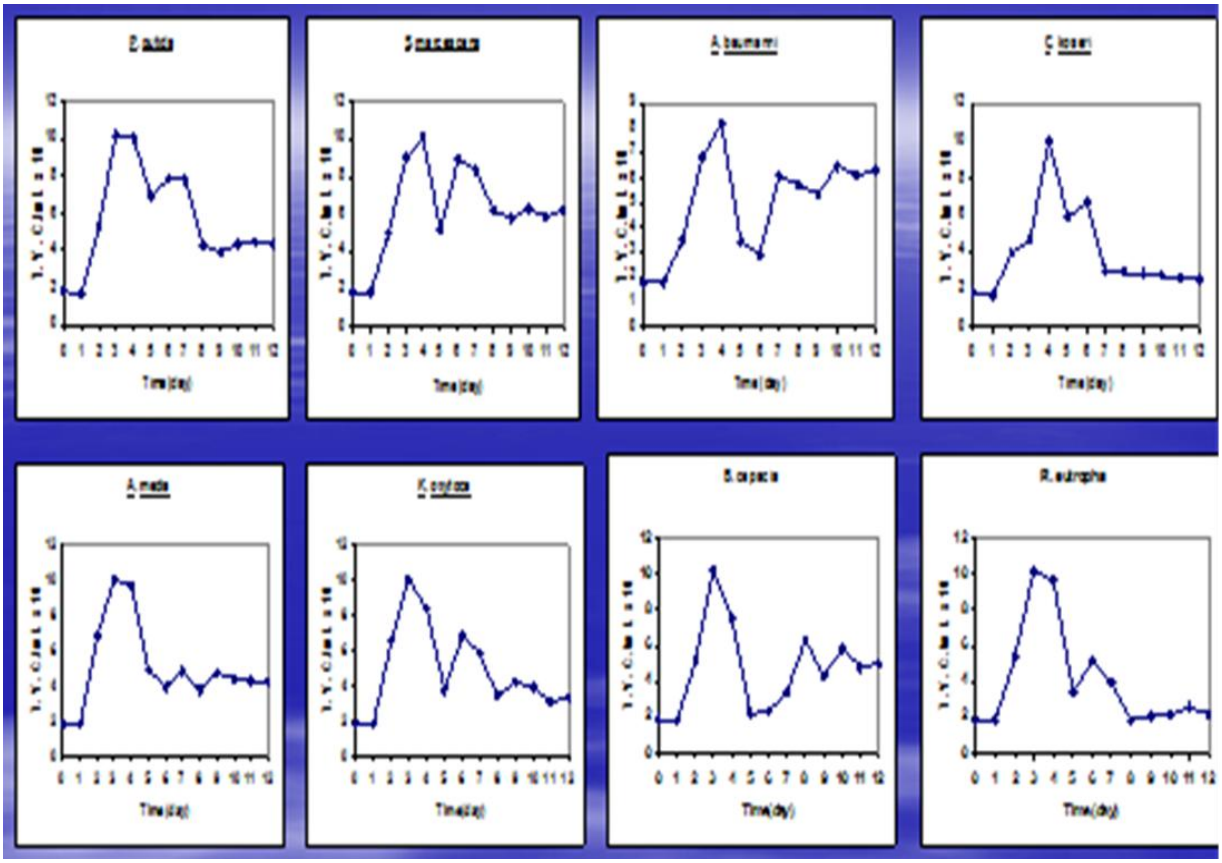

Figure (2) Growth of bacterial isolates in liquid mineral salts medium containing 10% of the chemical additive (concentrate) as a sole source of carbon and energy.

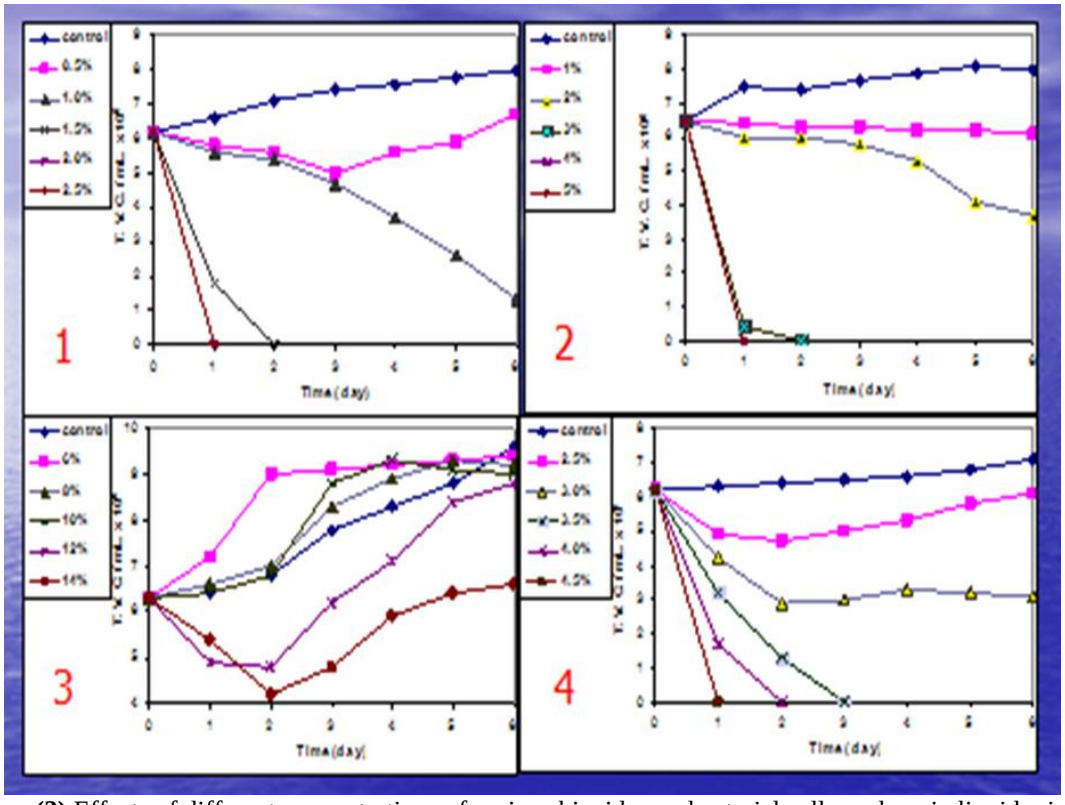

Figure (3) Effects of different concentrations of various biocides on bacterial cell numbers in liquid mineral salts medium containing 4% oil emulsion.

1- Chlorhexidine gluconate
2- Na- Tetraborate
3- Tween- 80
4- E.G.M.E.

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