Punjab Univ. J. Zool., Vol. 24 (1-2), pp. 49-60, 2009

SCREENING AND CHARACTERIZATION OF ALKALIPHILIC BACTERIA FROM INDUSTRIAL EFFLUENTS

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Abstract: The organisms which show potential to survive and work in acidic and alkaline environment play an important role in various industrial processes. This study was carried out to screen, isolate and characterize alkaliphilic bacteria from industrial wastes. For this purpose water and soil samples were collected from Chishtian and Quaid-e-Azam industrial area of Kott Lakh-Patt, Lahore. Screening of bacteria was carried out on LB agar plates at pH 8, 9 and 10. Out of 39 isolates, 20 were found to be alkaliphilic in nature. Glycerin stocks were prepared and they were characterized physically and biochemically for identification which revealed that they belong to genera *Alcaligenes*, *Escherichia*, *Natronobacterium*, *Aeromonas*, *Pseudomonas*, *Marinococcus*, *Neisseria*, *Micrococcus*, *Sporosarcina*, *Pleisomonas* and *Cupriavidus*. BCTL-147 isolate growing at pH 10, belonging to genus *Alcaligenes* was further characterized for optimum growth conditions (temperature and pH). The optimum pH was found to be 10 while optimum temperature for growth was 35°C. The growth curve of this isolate was prepared at optimum conditions. This high optimum pH bacterial isolate can further be tested for its biochemical potential and other significant properties.
Key words: extremophiles, basophilic microbes, industrially important

INTRODUCTION

Alkaliphilic bacteria are considered as subgrouping of extremophile microorganism. The enzymes from these organisms are extremely important for various biotechnological and industrial processes under very harsh alkaline conditions. The term “alkaliphile” is used for microorganisms that grow optimally at pH range above 9 but cannot grow or grow slowly near-neutral pH. Alkaliphiles include prokaryotes,

0079-8045/09/0049-0060 $ 03.00/0

eukaryotes, and archaea (Horikoshi, 1996, 1999, 2004). Microorganisms simply classified as alkaliphiles are mesophiles and consist of two main physiological groups: alkaliphiles and haloalkaliphiles (Horikoshi, 1999; Eichler, 2001; Wiegel and Kevbrin, 2004). They require an alkaline pH of 8 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH and high salinity (Gomes *et al*., 2004). The discovery of alkaliphiles was fairly recent and they have been isolated from neutral environments, sometimes even from acidic soil samples and feces. Haloalkaliphiles have been mainly found in extremely alkaline and saline environments, such as the Rift Valley lakes of East Africa and the Western Soda lakes of the United States. The viable counts of alkaliphiles are higher in samples from alkaline environments. The cell surface may play a key role in keeping the intracellular pH value between 7 and 8.5, allowing alkaliphiles to thrive in alkaline environments, although adaptation mechanisms have not yet been clarified (Horikoshi, 1996, 1999, 2004). Alkalithermophiles as well as alkaliphiles have also been isolated from alkali and hot environments such as alkaline hot springs, the new alkaline hydrothermal vents of the Lost City and alkaline lakes in Africa, Egypt and Israel (Gomes *et al*., 2004).

Alkaliphiles have made a great impact in industrial applications. Studies on these organisms have led to the discovery of many types of enzymes that exhibit interesting properties. Biological detergents contain alkaline enzymes, such as alkaline cellulases, proteases and lipases *etc.*, that have been produced from alkaliphiles (Fukumori *et al.*, 1985; Fujiwara and Yamamoto 1987; Fujiwara *et al.*, 1991). The current proportion of total world enzyme production utilized for the laundry detergent market exceeds 60% of the total enzyme requirements. Alkaline enzymes have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10 (Horikoshi, 1996, 1999, 2004). These enzymes are commercially available but their import requires lot of foreign exchange. Another important application is the industrial production of cyclodextrin by alkaline cyclomaltodextrin glucanotransferase. This enzyme has reduced the production cost and paved the way for cyclodextrins use in large quantities in foodstuff, chemicals, and pharmaceuticals. Alkaline proteases decompose the gelatinous coating of x-ray films, from which silver has been recovered later on (Fukumori *et al.*, 1985; Fujiwara and Yamamoto 1987; Fujiwara *et al.*, 1991). It has also been reported that alkali-treated

wood pulp could be biologically bleached by xylanases produced by alkaliphile bacteria. Polysaccharide degrading enzymes like amylases, pectinases and chitinases were also produced from alkaliphile microorganisms (Horikoshi, 1996, 1999, 2004). This study was carried out to screen, isolate and characterize the bacteria which can survive and work under extreme alkaline condition from local environment.

MATERIALS AND METHODS

Sample Collection

Industrial wastes samples (water and soil) for isolation of pure culture were collected in sterilized glass bottles from Chishtian and Quaid-e-Azam industrial area, Kott Lakh-Patt, Lahore. The location, physical status, pH and temperature of the soil and water sample at the time collection was shown in Table I.

Screening and isolation of bacteria

Two types of media (Lauria Bertani) LB agar and LB broth with three different pH values (8, 9 and 10) were used for this study. Serial dilutions of water and soil samples were prepared. From these dilutions each of samples (100µl) after shaking was spread over LB agar plates. The plates were then incubated at 37°C in an inverted position for 24 hours. Pure cultures were prepared and stored as glycerol stocks at 4°C for further studies.

Identification of bacteria

Different morphological, physical and biochemical tests were performed to identify the bacterial isolates which are given below.

Morphological and physical characterization

Morphological and physical characterization was carried out in terms of colour and morphology of bacterial colonies on LB agar plates, motility, endospore formation and Gram staining reaction. Tests for growth on nutrient agar, nutrient broth, E.M.B media on 2, 4 and 20% NaCl. McConkey test for gram negative enterobacteria was also performed.

Table I: The location, physical status, pH and temperature of the collected samples at collection site

Sr. no.	Sample collection sites	Physical form	Sample pH	Sample temperature (°C)	Isolate code
1	Morgan Bread Industry, Lahore	Liquid (water)	5	27	BCTL-153 (pH 8)
2	Dyeing Industry, Lahore	Liquid (water)	9.5	47	BCTL-154 (pH 8)
3	Noor Habib Dyeing Industry, Lahore	Liquid (water)	9-10	45	BCTL-145, 146, 147, 148, 149,150 (pH 10) BCTL-151, 152 (pH 9)
4	Al-quraish Bore Mills, Lahore	Liquid (water)	8	30	BCTL-138 (pH 10) BCTL-139, 140, 141, 142 (pH 9) BCTL-143, 144 (pH 8)
5	Pak Public Bore Mills, Chishtian	Liquid (water)	8	31	BCTL-116, 117, 118, 119, 120 (pH 10) BCTL-121, 122, 123, 124, 125, 126, 127 (pH 9) BCTL-128, 129, 130 (pH 8)
6	Al-Quraish Bore Mills, Lahore	Solid (Soil)	10	30	BCTL-131, 132 (pH 10) BCTL-133, 134, 135 (pH 9) BCTL-136, 137 (pH 8)

Biochemical characterization

Biochemical characterization includes starch hydrolysis, H_2S production, oxidase, catalase, indole, citrate utilization and antibiotic sensitivity tests.

Optimization of temperature for bacterial growth

In order to study the optimum temperature for bacterial growth, LB broth at pH 10 was prepared and poured (4.5ml) in 12 sets of test tubes (each set containing 4 test tubes *i.e.*, 3 replicates each and 1 control). After sterilization and cooling the tubes were inoculated with inoculums of bacterial isolate BCTL-147. The culture tubes were allowed to incubate at 25, 30, 35, 40 and 45°C. The light absorbance of cell biomass was recorded at 600nm after every half hour for 24 hours and plotted in graphical form (Fig. 1).

Optimization of pH for bacterial growth

In order to study the optimum pH for bacterial growth, LB broth having different pH (9.5, 10.0, 10.5 and 11.0) was prepared. The data was plotted in the form of growth curve (Fig. 2). The remaining procedure similar to above section.

Preparation of growth curve

The growth curve for *A. latus* was prepared in the LB broth using the optimum pH and temperature as worked out in the previous section (Fig. 3).

RESULTS

Screening and preparation of pure cultures

Water and soil samples were collected from Chishtian and Quaid-e-Azam industrial area of Kott Lakh-Patt, Lahore. Screening of alkaliphilic bacteria was carried out by spread plate method having alkaline pH 8, 9 and 10 from these samples. Thirty nine bacterial strains were isolated. They were given laboratory codes (Table I). Out of these 39 isolates, 20 showed significant growth in alkaline environment. These alkaliphiles were identified on the basis of their physical, morphological and biochemical characteristics (Table II).

Characterization of alkaliphilic isolates

Isolate with code number BCTL-147 and high pH (10.0) tolerances, identified as *Alcaligenes latus*, was tested further for characterization and optimization of its growth conditions.

Effect of temperature and pH on the growth of Alcaligenes latus

Figure 1 shows the effect of temperature change on the growth of *A. latus.* The optimum temperature for growth of *A. latus* was found to be 35°C as rapid growth was observed at this temperature. Away from the optimum temperature at both extremes *i.e.*, at 25ºC and at 40 and 45°C the growth was minimum with absorbance in the range of 25-30 (Fig. 1). Almost similar growth pattern was noticed at 30 and 35°C after 7 hours incubation. Stationary growth was noticed within 10 hours of incubation at 35°C while it took longer time almost (16 hours) to reach the stationary phase when incubated at 30°C. It was noticed that 35°C was most suitable temperature for growth and to get suitable product within minimum time period (Fig. 1).

The optimum pH for growth of *A. latus* was found to be 10 at optimum temperature *i.e.,* 35°C, however growth was quite remarkable in the pH range of 10-11. After 10 hours of incubation there was a slight difference in growth at pH 10 and 11 but maximum growth was observed in pH 10 (Fig. 2).

Growth curve for *A. latus* was plotted under optimum growth conditions of temperature (35°C) and pH (10.0). The curve achieved mone growth level then achieved in case of temperature and pH curves. The maximum turbidity (absorbance) shown by this growth curve was 0.6, while in case of figure 1 the maximum absorbance was 0.45.

Antibiotic sensitivity testing

Alcaligenes latus was tested for antibiotic sensitivity which showed resistance against carbenicillin, furazolidone, penicillin and zinacef. On the other hand, inhibition (clear zones) were observed for gentamicin, norfloxacin, neomycin, oxytetracycline and tobramycin that showed the sensitivity for these antibiotics. The results showed that *A. latus* was highly sensitive against gentamicin.

Table II: Identification of screened alkaliphilic bacterial isolates collected from various polluted areas

S. No.	Isolate code	Genus/species
1	BCTL-116	*Vibrio cholerae*
2	BCTL-119	*Escherichia sp.*
3	BCTL-121	*Planococcus kocurii*
4	BCTL-122	*Aeromonas eucrenophila*
5	BCTL-124	*Pseudomonas aeruginosa*
6	BCTL-125	*Pseudomonas sp.*
7	BCTL-126	*Marinococcus halophilus*
8	BCTL-131	*Neisseria denitrificans*
9	BCTL-132	*Alcaligenes sp.*
10	BCTL-133	*Planococcus sp.*
11	BCTL-134	*Micrococcus roseus*
12	BCTL-135	*Aeromonas sp.*
13	BCTL-137	*Alcaligenes latus*
14	BCTL-139	*Natranobacterium gregoryi*
15	BCTL-140	*Sporosarcina ureae*
16	BCTL-143	*Pleisomonas shigelloides*
17	BCTL-144	*Cupriavidus nector*
18	BCTL-147	*Alcaligenes latus*
19	BCTL-148	*Neisseria sp.*
20	BCTL-153	*Neisseria sp.*

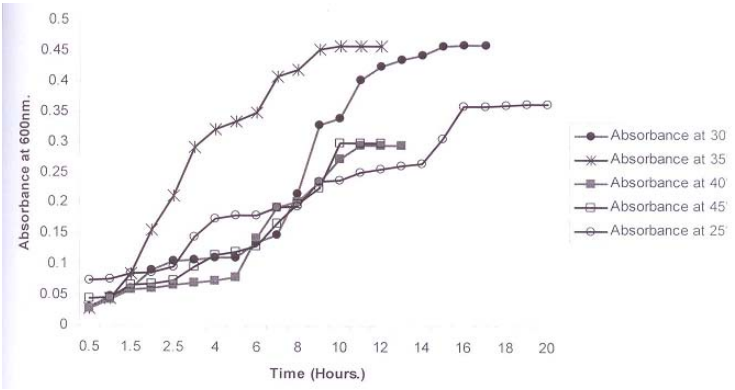

Fig. 1 Effect of temperature on the growth of *Alcaliagines latus.*

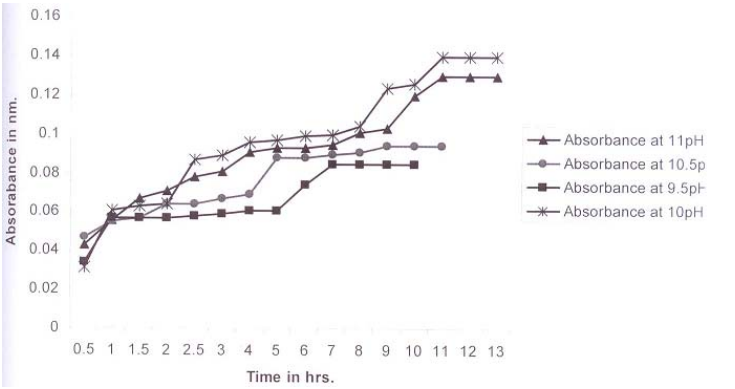

Fig. 2 Effect of pH change on the growth of *Alcaliagines latus* strain.

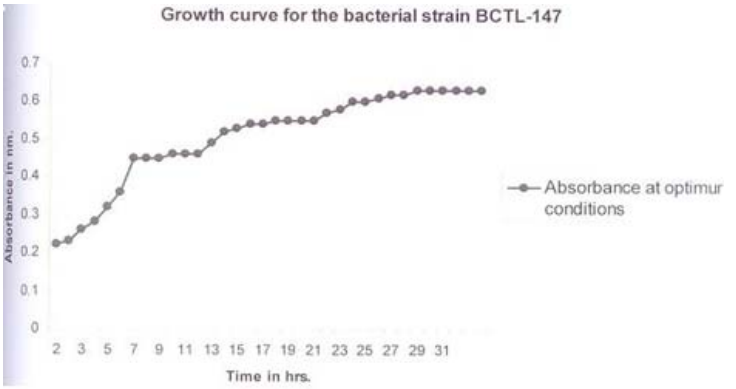

Fig. 3 Growth curve of *A. latus* strain under optimum conditions.

DISCUSSION

Extremophiles are found in a wide variety of environment in soil, air and water bodies. Few examples of harsh environment where they found are salt flats, hydrothermal vents and in subsurface ice (Gomes and Steiner, 2004). The classification of extremophiles is based on the conditions in which they are living (Duncan *et al*., 2005). In the present study the extremophile bacteria growing successfully in alkaline environment were screened, isolated followed by preparation of pure cultures. These bacterial isolates were identified on the basis of different morphological, physical and biochemical tests. Some of these isolates were reported to be pathogenic. Most isolates of *Neisseria* sp. have been reported to lack capsule, oxidize glucose and maltose (Yazdankhah *et al*., 2004) which is in accordance with present study. These bacteria cannot be maintained under excess glucose conditions. It has been reported that more variety of products can be produced and amino acid catabolism was shown by this organism can be observed under low glucose environment. This mixed and simultaneous utilization of substrate was responsible for higher yield and species diversity (Mchkee *et al.*, 1985). The isolates BCTL-131, BCTL-148, BCTL-153 belong to *Neisseria* sp. These bacteria are gram positive, motile, glucose oxidizing, non endospore forming cocci. The isolate BCTL-131 was identified as *N. denitrificans*, which is highly nitrogen reducing species (Xin *et al.*, 2001). Similarly BCTL-121 (*Planococcus kocurii)* isolate growing at pH 9.0, having yellow colonies can be commercially important because of its growth at 20% salinity (Miller, 1985). Other *Planococcus* sp. have been reported to accumulate glycine-betaine with glutamate and an unknown compound (Romano *et al*., 2003). The bacterial isolates BCTL-122 and BCTL-135 were identified as *Aeromonas* sp. and BCTL-143 was identified as *Pleisomonas* sp. These isolates were collected from bore mills effluents. The studies have demonstrated that *Aeromonas* sp. and *Pleisomonas* sp. are universally distributed and widely isolated from clinical environment and food samples. The most important property proved in literature is that they can grow rapidly at +4ºC (Wadstrom and Ljungh, 2002). *Aeromonas* sp. have the potential to decolorize azo deyes (Hayase *et al.*, 2000). *Aeromonas* sp. have been reported to secrete wide variety of enzymes like lipases, proteases, chitinases, nucleases and amylases, involved in pathogenicity

and environmental adaptability. The isolate BCTL- 124 and BCTL-125 showed orange colonies were identified as *Pseudomonas aeruginosa*. This species have demonstrates the ability to mineralize a higher concentration of pentachlorophenol and also well known as a candidate in environmental biodegradation (Radehaus *et al.,* 1992).

The isolate BCTL-116 was identified as *Vibrio cholerae*. It was isolated from paper bore mills from Chishtian area. Ogg *et al.* (1989) isolated *Vibrio cholerae* from local swabs and freshly voided faces collected from 20 species of aquatic birds in Colorado and Utah during 1986 and 1987. The isolate BCTL-126 identified as *Marinococcus halophilus* was a gram positive, moderate, halophilic eubacterium (Louis and Galinski, 1997). The isolate BCTL-144 was identified as *Cupriavidus necator,* is an aerobic, gram negative bacteria which is in accordance with the study of Sato *et al.* (2006). The isolate BCTL-139 was identified as *Natronobacterium gregory* which showed significant growth even at 20% NaCl concentarion. These type of organisms can be exploited commercially in high salinity is required in media (Xin *et al.,* 2001). This characteristic of high salinity tolerance is also shown by *Planococcus kocurii* (Miller, 1985). Isolate BCTL-144 was a *Micrococcus roseus* with orange colonies. They possesed esterase activity and hydrolyzed *p*-nitrophenyl derivatives of fatty acids. *M. roseus* has been shown to produce bicyclic keto-carotenoid compounds in the fermentation medium (Cooney and Berry, 1981). Enterases are the enzymes involved in breakdown of several types of phosphate esters, xenobiotics and also in development of tolerance/resistance in animals.

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(Received: 20 July, 2009; Revised: 11 November, 2009)