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DETERMINATION OF THE GENETIC MARKER OF THE MUTAGENIZED STRAINS OF *PSEUDOMONAS AERUGINOSA* AND *BACILLUS CEREUS* ISOLATED FROM EFFLUENT OF PETROLEUM REFINERY

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ABSTRACT
Bacillus cereus and Pseudomonas aeruginosa were isolated from the effluent of the Kaduna refining and petrochemical company, using standard methods. These were UV-irradiated for 30mins and thereafter subjected to nitrous acid treatment. Determination of essential amino acids required by both parents and mutants for growth showed that the mutant of Pseudomonas aeruginosa did not require valine and histidine for growth. The mutational treatments resulted in a sense mutation with beneficial effects of increased petroleum degradation. The amino acids requirement for growth could thus be used as a genetic marker for organisms that are subjected to mutational treatments.

Keywords: Mutation, Marker, Organisms, Petroleum, Degradation.

INTRODUCTION

Mutation is a stable heritable change in the DNA sequence. Most mutations harm an organism but some could be beneficial (Liu, 2005; Anon. 2007b, c). Mutations are of two types basically - base pair (nucleotide pair) substitutions and frame shift mutation. These could either be spontaneous or induced. Spontaneous mutation occurs as a result of natural processes in cells and have very low frequency of occurrence (10^{-12}) while induced mutation results from the exposure of an organism to a mutagen which is a natural or human-made agent (physical or chemical) that can alter the structure or sequence of DNA (Anon., 2007a, b; Prescott *et al*., 2008). Chemical mutagens can, on the basis of mode of action, be either base analogs e.g. bromouracil and amino purine; chemicals which alter structure and pairing properties of bases e.g. nitrous acid, nitrosoguanidine, methylmethane sulfonate (MMS) and ethylmethane sulfonate (EMS); intercalating agents e.g. acridine orange, proflavin and ethidium bromide or agents altering DNA structure e.g. NAAAF (N-acetoxy-2-acetylamine fluorene), psoralens and peroxides. Physical agents include irradiations which could be either ionizing e.g. X – and gamma-rays or non-ionizing e.g. UV irradiation. Ionizing radiation produces a range of effects on DNA both through free radical effects and direct action through breaks in one or both strands, damage or/loss of bases (mutation) and cross linking of DNA to itself or proteins. UV (Ultraviolet) irradiation is less energetic but its wavelengths are preferentially absorbed by bases of DNA and by aromatic amino acids of proteins. Killing of cells by UV irradiation is due primarily to its action on DNA and UV irradiation at 260nm is most effective as a lethal agent (Anon., 2007a). UV light causes dimerization of two adjacent pyrimidine residues (Prescott *et al.,* 2008).

As DNA damage occurs spontaneously and also as a result of ubiquitous environmental agents, most organisms possess some capacity to repair the DNA. Therefore, the mutagenic treatment of organisms does not necessarily result in mutants being produced (Prescott *et al.,* 2008). The establishment of mutation in treated organisms is often difficult. Various methods have been employed to this end.

Organisms require essential (or limiting) amino acids. Pontecorvo (1949) employed essential amino acids in auxanographic studies and obtained varying growth–factor requirements for parents and mutants. The aim of this research is to determine the amino acid requirements as a genetic marker for mutagenized strains of *Pseudomonas aeruginosa* and *Bacillus cereus* isolated from the effluent of Kaduna refining and petrochemical company, Kaduna, Nigeria.

MATERIALS AND METHODS

Source of sample: The refinery effluent was obtained from the Kaduna refining and Petrochemical Company, Kaduna at the point of discharge into the Romi stream. Sample was transported in ice chest, to arrest microbial growth, to the Microbiology laboratory at Ahmadu Bello University, Zaria.

Isolation of the Bacillus sp and *Pseudomonas* sp*:* Refinery effluent samples were inoculated using pour plate method on J-Medium (Tryptone 5g, Yeast Extract 15g, K_2PO_4 3g, Glucose 2g, Agar 20g, Distilled water I litre, pH 7.4) for the isolation of *Bacillus* sp and on King's Medium B (Proteose peptone 2g, Glycerol 1ml, K_2HPO_4 0.15g$MgSO_4$ 15g, Agar 1.5g, Distilled water 100ml, pH 7.2) for the isolation of *Pseudomonas* sp (Anon., 2007e). The inoculated plates were incubated at 37^{o}C for 24h.

The isolates were identified in accordance with the methods reported by Cowan and Steel (2004). The following were carried out: a.*Cultural characteristics:* The growth of the organisms on nutrient agar, MacConkey agar and blood agar were observed including pigment formation and odor of the colonies.
b. *Aerobic growth:* The growth of the organism on the agar surface of nutrient agar was recorded as aerobic growth.
c. *Anaerobic growth:* The inoculated plates were incubated in anaerobic jars containing anaerobic GasPak at 37^{o}C for 24h and observed for growth after incubation.
d. *Fluorescence under UV light:* The inoculated plates with growth on nutrient agar were viewed under UV light at 254nm for fluorescence.
e. *Gram staining*: This was carried out in accordance with procedure in Cowan and Steel (2004).
f*. Endospore test*: This was carried out in accordance with procedure in Cowan and Steel (2004).
g. *Motility test:* This was carried out in accordance with procedure in Cowan and Steel (2004).
h. *Catalase test:* This was carried out in accordance with procedure in Cowan and Steel (2004).
j. *Oxidase test:* This was carried out in accordance with procedure in Cowan and Steel (2004).
k. *Oxidative-fermentative (OF) test:* This was carried out in accordance with procedure in Cowan and Steel (2004).
l. *Glucose, Mannitol, Lactose fermentation tests:* This was carried out in accordance with procedure in Cowan and Steel (2004).
m. *Citrate utilization test:* This was carried out in accordance with procedure in Cowan and Steel (2004).

Mutagenic treatment of the isolated *Bacillus* sp and *Pseudomonas* sp

Mutation with UV irradiation at 254nm: This was carried out by using a modification of the procedure reported by Ado (2004). The organisms were grown in nutrient broth for 24h and their microbial counts were determined. Ten milliliters of broth of each organism was aseptically transferred into separate sterile Petri dishes and placed at 6cm from the source of UV light for 30mins in a dark room. The UV irradiated organisms were then transferred into a sterile twenty milliliter test tube in a dark room and treated with 0.2% (w/v) caffeine and allowed to stand at room temp in the dark for 5h. The irradiated cells were then centrifuged at 1500rpm for five minutes, re-suspended in normal saline and re-centrifuged and discarded the supernatant. The treated organisms were then incubated at 18^{o}C for 16h and their microbial counts were determined.

Mutation with nitrous acid: This was carried out by using the procedure reported by Ado (2004). The organisms were grown in nutrient broth for 18-24h and their microbial counts were determined. To fifty milliliters of 50:50 organism: acetate buffer (0.2M, pH 4.4) suspension in a 150ml flask was added 1.5ml of membrane filter (0.2µm pore size) (GelmanSciences. Product No. 6224192) sterilized aqueous 2.0M sodium nitrate. This was allowed to stand at room temp for twenty minutes. The reaction was terminated by serial dilution with Tris HCl. The treated organisms were inoculated, using pour plate technique, on nutrient agar and incubated at 37^{o}C.

Determination of the amino acid requirements of parents and modified strains: This was carried out in accordance with the procedure reported by Pontecorvo (1949). A minimal medium (Glucose 25g, $NaNO_3$ 15g, $MgSO_4.7H_2O$ 1.3g, KCl 1.3g, KH_2PO_4 3.8g, $FeSO_4.7H_2O$ 10mg, $ZnSO_4$.$7H_2O$ 1.0mg, Agar 37.5g, Distilled water 2.5litres) was prepared and the pH adjusted from 3.08 to 6.93. This was then dispensed into sterile 20ml test tubes. Seven essential amino acids (histidine, leucine, lysine, methionone, phenylalanine, tryptophan and valine) and four organisms (B, $B_{UVNA,}$ P and P_{UVNA}) were tested. To a set of four test tubes was added each amino acid and each tube was inoculated with one test organism. A control was prepared containing all the amino acids. The inoculated tubes were incubated at 37^{o}C for 24h and the broths were observed for growth. Cloudy broths were recorded as positive utilization of the test amino acid.

Another set of test tubes containing minimal medium whose pH was adjusted from 3.08 to 7.63 was prepared. To each set of four test tubes were added all the tested amino acid except one and inoculated each of the test tubes in the set with the test organism. A control was prepared that contained all the tested amino acids. The inoculated tubes were incubated for 24h at 37^{o}C. Cloudy broths were recorded as positive.

RESULTS AND DISCUSSION

The determinations of the required essential amino acids for growth are presented in Tables 1 and 2. It was observed that *Pseudomonas aeruginosa* that was irradiated with UV light and then subjected to nitrous acid treatment (P_{UVNA}) did not require histidine and valine for growth while *Bacillus cereus* that was irradiated with UV light and then subjected to nitrous acid treatment (B_{UVNA}) and both parents (B and P) required all the seven tested essential amino acids – Leu, Lys, Try, Met, His, Val, and Phe - for growth (Table 1). However, all the organisms grew on the minimal medium that contained all the test amino acids less one. They also grew on the minimal medium that contained all the test amino acids (Table 2).

These show that there was a change in the gene sequence of the mutant P_{UVNA} that resulted in a sense mutation as in the genetic code, GUU, GUC, GUA and GUG code for valine (a synonymous amino acid) while CAU and CAC code for histidine. The mutational treatments meted out thereby led to a sense mutation as the amino acid required for growth differed in the parents and mutants.

These results agree with previous reports that any change in the gene sequence would affect the codons and ultimately the amino acid(s) produced (Pontacorvo, 1949; Prescott *et al.,* 2008; Anon., 2009).

Conclusion

The organisms isolated from the Kaduna Refining and Petrochemical Company, Kaduna and identified as *Bacillus cereus* and *Pseudomonas aeruginosa,* subjected to UV-irradiation for 30mins and followed by treatment with nitrous acid resulted in a sense mutation. The mutants had varied essential amino acids requirements for growth from their parents.

Table 1: Growth on minimum medium containing only the test amino acid

Amino acid	B	B_{UVNA}	P	P_{UVNA}
Histidine	+	+	+	-
Leucine	+	+	+	+
Lysine	+	+	+	+
Methionine	+	+	+	+
Phenylalanine	+	+	+	+
Tryptophan	+	+	+	+
Valine	+	+	+	-
MBM	+	+	+	+

Key: B = *Bacillus cereus;* B_{UVNA} = UV irradiated nitrous acid treated *Bacillus cereus;* P = *Pseudomonas aeruginosa;* P_{UVNA} = UV irradiated nitrous acid treated, *Pseudomonas aeruginosa;* + = growth; - = no growth

Table 2: Growth on minimal basal medium containing all the amino acids except the test amino acid

Amino acid	B	B_{UVNA}	P	P_{UVNA}
His^-	+	+	+	+
Leu^-	+	+	+	+
Lys^-	+	+	+	+
Met^-	+	+	+	+
Phe^-	+	+	+	+
Try^-	+	+	+	+
Val^-	+	+	+	+
All tested aas	+	+	+	+

Key: B = *Bacillus cereus;* B_{UVNA} = UV irradiated nitrous acid treated *Bacillus cereus;* P = *Pseudomona aeruginosa;* P_{UVNA} = UV irradiated nitrous acid treated *Pseudomonas aeruginosa;* + = growth; - = all other amino acids except the amino acid and aas = amino acids.

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