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Isolation and Growth Kinetic Studies Of Bacillus Methylotrophicus P10 & P11 From Godavarikhani Open Cast – Iii Coal Mine Soil Of The Singareni Collieries, Andra Pradesh.

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ABSTRACT
The study deals with isolation and identification of bacteria from fresh coal mine soil collected from seam three of the opencast coal mine with a temperature range of 35-50ºC and growth curve analysis of these isolates. Two strains of bacteria were isolated and they were identified as Bacillus methylotrophicus p10 and p11.Their growth analysis indicated normal optimal growth curve for both the isolates at 35ºC. However with increase in temperature, both the isolates showed deviated growth curve with reduction in the exponential phase and specific growth rate. The study concludes that both the isolates are mesothermophilic with capacity to tolerate higher temperature regime.

Keywords: *Bacillus methylotrophicus, Growth curve, Temperature, Coal mine soil.*

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I. INTRODUCTION

Soil contains phylogenetic groups of bacteria that are globally distributed and abundant in terms of the contributions of individuals of those groups to total soil bacterial communities. However, only a few bacteria such as Thiobacillus sp. and Methanogene sp. have been reported to live in the soil of coal mines [1]. Thiobacillus ferrooxidans was the first organism isolated from acidic bioleaching environment. It shares its environmental niche with other acidophilic bacteria which have a similar physiology and which can directly or indirectly compete for available inorganic substrates. It is therefore impossible as yet to define the precise role and importance of each organism in these dynamic populations, as the intimate link between microbial physiology and sulfide bio hydrometallurgy is incompletely understood [2] [3].

The microbiological studies on coal mine spoil overburdens of Basundhara coal field area of Mahanadi coal field limited, Orissa revealed the isolation of thermo and pH resistant Gram negative bacilli and cocci in a major proportion of total colony forming units of bacterial population in the fresh coal mine spoil. There have been also reports about the prevalence of Gram negative bacteria from coal mine spoils of different geographical regions [4],[5]. Our earlier microbiological studies in the soil of Godavarikhani Open Cast Project – III coal mine (Ramagundam Area) of Singareni Collieries Company Limited situated in Karim Nagar District of Andhra Pradesh revealed the presence of three Gram positive Bacillus bacteria [6]. The abundance, composition, and diversity of microbial communities within soils are strongly depth dependent [7]. The characterization of the microbial community within a soil sample is a very useful tool in determining the overall health of the soil. Measurement of the soil microbial community may certainly be used to determine biodiversity, ecological processes and structures. That microbial measurement has utility as an indicator of the re-establishment of connection between the biota and restoration of function in degraded systems. A comprehensive determination of soil microbial community characteristics is one way of approach for the success of restoration processes. Characterization is a very broad term that can cover many aspects of the soil microbes [2]. So, this present study bacteria found in the soil sample collected from seam three of open cast coal mine were identified and their growth pattern was analyzed.

II. MATERIALS AND METHODS

2.1. Study area

Godavarikhani Open Cast Project – III coal mine (Ramagundam Area) of Singareni Collieries Company Limited is situated in Karim Nagar District of Andhra Pradesh. Geographically Godavarikhani is located at 18.8000° N 79.4500° E. It has an average elevation of 179 meters (590 feet) and is situated in the Godavari Valley coalfields.

2.2. Collection of Samples and Isolation of Bacteria

The soil samples were collected in sterile vials from the bottom layer of the third seam. The diluted samples were plated onto isolation media (LB agar) by pour plate method and incubated at 37^{o} C for 24 hours. Sub-culturing was done by streak plate method taking the isolated colonies of bacterial cultures and again incubated at 37^{o} C for 24-48 hrs.

2.3. Identification of Bacteria

Identification of the selected isolates Bio-Chemically [8] and also carried out using 16s rDNA ribotyping .

2.4. Nucleic acid extraction and purification

10ml of overnight grown bacterial culture was transferred into 5 eppendorf tubes and centrifuged for 5 min at 5000 rpm. Supernatant was discarded. Pellet was resuspended in 1ml of extraction buffer by pipetting up-and-down repeatedly. Suspension was transferred to a sterile 2-ml microcentrifuge tube and centrifuged for 10 min at 10000 rpm.300 µl of both phenol and chloroform/isoamyl alcohol was added to the pellet and centrifuged for 3min at 10,000 rpm or until phases were well separated. With a sterile pipette tip, aqueous phase was transferred to a new 2 ml tube. 500 µl of chloroform was added to supernatant.

2.5. Characterization of bacteria using 16s rDNA typing

2.5.1. PCR Amplification of the 16s rDNA gene [9],[10].

49 µl of the "PCR mix" was pipetted in ice bucket into the 0.2 mL microcentrifuge tube. The PCR mix contains the forward and reverse primers, dNTPs, Taq polymerase, $MgCl_2$ and PCR reaction buffer. 1µL of PCR mix was added to the cell solution.

We used the following universal bacterial primers: 16s Forward (5-AGAGTTTGATCATGGCTCAG-3) and 16s Reverse (5-GGTTACCTTGTTACGACTT-3) was used to characterize the unknown bacterial species. 49 µl of the above mix was added to 1µl of prepared template DNA per PCR reaction.

2.5.2. DNA Sequencing

In our sequencing reactions, we used dideoxynucleotides labeled with different colored fluorescent tags. Also, in DNA sequencing only one primer was used, so only one of the two strands was used as a template in the sequencing reaction. Once the results of the DNA sequencing are known, we were able to search the database of known sequences for a match to this sequence. The resulted sequence was compared to the Gen Bank database at the National Centre of Biotechnological Information (NCBI) by using BLAST (Basic Local Alignment Search Tool) for sequences similarity.

2.6. Growth kinetics studies

Pure cultures of Gram positive bacilli bacteria (Bacillus methylotrophicus strain p10 & p11) were isolated from the soil samples were used for the present study. A loop of bacteria from the pure culture slant of each bacterial isolate was aseptically transferred to a sterilized 250mL Erlenmeyer flask containing 100mL of nutrient broth (peptone-5g/L, Beef extract-3g/L and NaCl-5g/L) and was activated by incubating for 24hrs at 35ºC. Fifteen number of Erlenmeyer flasks (capacity: 150mL) and each containing 50mL of sterilized nutrient broth were taken and to the 12 number flasks, one ml of activated Gram ($^{+}$ve) Bacillus methylotrophicus strain p10 culture was aseptically transferred. These flasks were then incubated in a shaker incubator over a temperature range of 35 to 50ºC for a period of 285-300 minutes. Simultaneously, three number of Erlenmeyer flask (capacity: 150mL) having only nutrient broth (uninoculated) were taken as control. The culture content of the flasks were spectrophotometrically analyzed for growth of the bacteria at 640nm with respect to different time (15 minutes) interval. Same procedure was adopted for the growth assessment of another Gram positive Bacillus methylotrophicus strain p11 isolate.

Specific growth rate (µ) of the bacterial isolates at different temperature was calculated as follows [11]:

$$\frac{\log N_t - \log N_o}{t_1 - t_0} = \frac{\mu}{2.303}$$

Where,

N0 = Absorbance at initial of the exponential phase of growth

Nt = Absorbance at end of the exponential phase of growth

$t_1 - t_0$ = Time difference to achieve absorbance from N0 to Nt

III. RESULTS

During this study, two bacterial isolates, i.e. Bacillus methylotrophicus strains p10 and p11 were isolated from soil from the Godavarikhani Open Cast Project – III coal mine (Ramagundam Area) of Singareni

Collieries Company Limited is situated in Karim Nagar District of Andhra Pradesh, India, by using LB agar medium culture. The molecular identification of 16SrRNA gene sequences showed that the isolates had 99% similarity to genus Bacillus sp. Sequence analysis of the 16S rRNA genes of 3 representative strains revealed that all of the strains were closely related to strains which have been sequenced previously and also confirmed the phylogenetic diversity of bacteria present in coal mining environments. The identified sequences were deposited in GenBank (Accession Number: KM434241 – KM434242).

3.1. Characters of B. methylotropicus P10 and P11

Bacillus methylotropicus is Gram-positive, endospore-forming, strictly aerobic, motile rods (0.63–0.6461.8–2.7 mm) occurring singly or in pairs. Colonies are creamy white, convex, translucent with regular edges, slow-growing and 0.2–0.8 mm in diameter. Catalase, oxidase, pectinase and protease activities are positive. Cellulase, arginine dihydrolase, urease and b-galactosidase activities are absent. Nitrate reduction and hydrolysis of gelatin, are positive. Tests for glucose fermentation and indole production are negative. Methanol, trimethylamine and ethanol are utilized as sole carbon sources. Ammonium sulfate, potassium nitrate, sodium nitrate, ammonium chloride, L-alanine, L-glutamine, L-tryptophan, glycine, potassium cyanate and potassium thiocyanate are utilized as sole nitrogen sources, but urea, methylamine, L-glutamate, diphenylamine and L-aspartic acid are not utilized as nitrogen sources.

Table 1. Differential Characteristics Of Strain P10 & P11

Characteristic/Biochemical Test	**Bacillus methylotropicus**	
	Strain P10	**Strain P11**
Pigmentation	creamy white	creamy white
Gram Staining	+	+
Endospore-forming	+	+
Aerobic	Strictly aerobic	Strictly aerobic
Motility	Motile Rod	Motile Rod
Catalase	+	+
Oxidase	+	+
Pectinase	+	+
Protease	+	+
Cellulase	-	-
Arginine dihydrolase	-	-
Urease	-	-
B-galactosidase	-	-
Nitrate reduction	+	+
Hydrolysis of gelatin	+	+
Glucose fermentation	-	-
Indole production	-	-
Utilization of Methanol,	+	+
Trimethylamine	+	+
Ethanol	+	+
Utilization of Ammonium sulfate	+	+
Potassium nitrate	+	+
Sodium nitrate	+	+
Ammonium chloride	+	+
L-alanine	+	+
L-glutamine	+	+
L-tryptophan	+	+
Glycine	+	+
Potassium cyanate	+	+
Potassium thiocyanate	+	+
Urea	-	-
Methylamine	-	-
L-glutamate	-	-
Diphenylamine	-	-
L-aspartic acid	-	-

3.2. Bacillus methylotrophicus strain P10 16S ribosomal RNA gene, partial sequence

GenBank: KM434241.1

1 gggtgagtaa cacgtgggta acctgcctgt aagactggga taactccggg aaaccggggc
61 taataccgga tggttgtttg aaccgcatgg ttcagacata aaaggtggct tcggctacca
121 cttacagatg gacccgcggc gcattagcta gttggtgagg taacggctca ccaaggcgac
181 gatgcgtagc cgacctgaga gggtgatcgg ccacactggg actgagacac ggcccagact

241 cctacgggag gcagcagtag ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc
301 cgcgtgagtg atgaaggttt tcggatcgta aagctctgtt gttagggaag aacaagtgcc
361 gttcaaatag ggcggcacct tgacggtacc taaccagaaa gccacggcta actacgtgcc
421 agcagccgcg gtaatacgta ggtggcaagc gttgtccgga attattgggc gtaaagggct
481 cgcaggcggt ttcttaagtc tgatgtgaaa gcccccggct caaccgggga gggtcattgg
541 aaactgggga acttgagtgc agaagaggag agtggaattc cacgtgtagc ggtgaaatgc
601 gtagagatgt ggaggaacac cagtggcgaa ggcgactctc tggtctgtaa ctgacgctga
661 ggagcgaaag cgtggggagc gaacaggatt agataccctg gtagtccacg ccgtaaacga
721 tgagtgctaa gtgttagggg gtttccgccc cttagtgctg cagctaacgc attaagcact
781 ccgcctgggg agtacggtcg caagactgaa actcaaagga attgacgggg gcccgcacaa
841 gcggtggagc atgtggttta attcgaagca acgcgaagaa ccttaccagg tcttgacatc
901 ctctgacaat cctagagata ggacgtcccc ttcgggggca gagtgacagg tggtgcatgg
961 ttgtcgtcag ctcgtgtcgt gagatgttgg gttaagtccc gcaacgagcg caacccttga
1021 tcttagttgc cagcattcag ttgggcactc taaggtgact gccggtgaca aaccggagga
1081 aggtggggat gacgtcaaat catcatgccc cttatgacct gggctacaca cgtgctacaa
1141 tggacagaac aaagggcagc gaaaccgcga ggttaagcca atcccacaaa tctgttctca
1201 gttcggatcg cagtctgcaa ctcgactgcg tgaagctgga atcgctagta atcgcggatc
1261 agcatgccgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac accacgagag
1321 tttgtaacac ccgaagtcgg tagggtaa

3.3.Bacillus methylotrophicus strain P11 16S ribosomal RNA gene, partial sequence
GenBank: KM434242.1

1 tctgacggag caacgccgcg tgagtgatga aggttttcgg atcgtaaagc tctgttgtta
61 gggaagaaca agtgccgttc aaatagggcg gcaccttgac ggtacctaac cagaaagcca
121 cggctaacta cgtgccagca gccgcggtaa tacgtaggtg gcaagcgttg tccggaatta
181 ttgggcgtaa agggctcgca ggcggtttct taagtctgat gtgaaagccc ccggctcaac
241 cggggagggt cattggaaac tggggaactt gagtgcagaa gaggagagtg gaattccacg
301 tgtagcggtg aaatgcgtag agatgtggag gaacaccagt ggcgaaggcg actctctggt
361 ctgtaactga cgctgaggag cgaaagcgtg gggagcgaac aggattagat accctggtag
421 tccacgccgt aaacgatgag tgctaagtgt tagggggttt ccgccccta gtgctgcagc
481 taacgcatta agcactccgc ctggggagta cggtcgcaag actgaaactc aaaggaattg
541 acgggggccc gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc gaagaacctt
601 accaggtctt gacatcctct gacaatccta gagataggac gtcccttcg ggggcagagt
661 gacaggtggt gcatggttgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa
721 cgagcgcaac ccttgatctt agttgccagc attcagttgg gcactctaag gtgactgccg
781 gtgacaaacc ggaggaaggt ggggatgacg tcaaatcatc atgcccctta tgacctgggc
841 tacacacgtg ctacaatgga cagaacaaag ggcagcgaaa ccgcgaggtt aagccaatcc
901 cacaaatctg ttctcagttc ggatcgcagt ctgcaactcg actgcgtgaa gctgga

Pure cultures of Gram positive bacilli bacteria (Bacillus methylotrophicus strain p10 & p11) isolated from the soil samples were used for the present growth kinetics study.

Fig.1illustrates the growth curve of Gram positive Bacillus methylotrophicus **strain p10** at a temperature regime of 35 to 50ºC. The bacteria showed a lag phase up to 60 to 90 minutes. The exponential phase for the bacteria growth at 35ºC lasted up to 195 minutes, after which the bacteria entered in to stationary phase. However, the growth patterns of the bacteria at 40, 45 and 50ºC were marked to be different. Growth at 40ºC showed relatively earlier culmination of exponential phase. At 45ºC, although exponential phase did extend up to 225 minutes, the slope of the phase was much less compared to 35ºC or 40ºC. At 50ºC, the bacteria did exhibit little growth showing still less slope during the exponential phase.

Fig. 2 illustrates the growth curve of Gram positive Bacillus methylotrophicus **strain p11** over a temperature regime of 35 to 50ºC. The bacteria showed a lag period up to 90 to 135 minutes. The exponential phase for the bacteria growth at 35ºC lasted up to 195 minutes of incubation time, there after the stationary phase started. Whereas at 40ºC, the bacteria exhibited relatively earlier culmination of the exponential phase. However at 45ºC the bacteria did exhibit little growth and the slope of the phase was much less than 35 and 40ºC. In response to 50ºC the growth of bacteria was found to be insignificant.

Specific growth rate (μ) of the bacteria at different temperature (Fig. 3) indicated highest value of "μ" at 35ºC and the value decreased with gradual increase in the temperature

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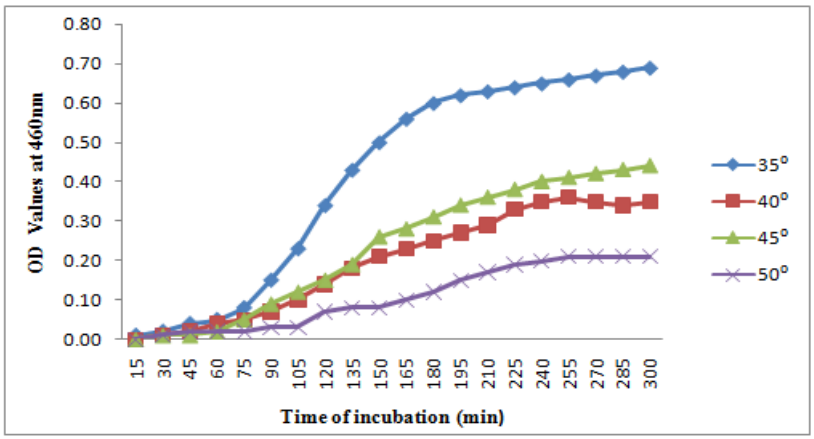

Figure 1: Growth curve of Bacillus methylotrophicus **strain p10** at different temperature

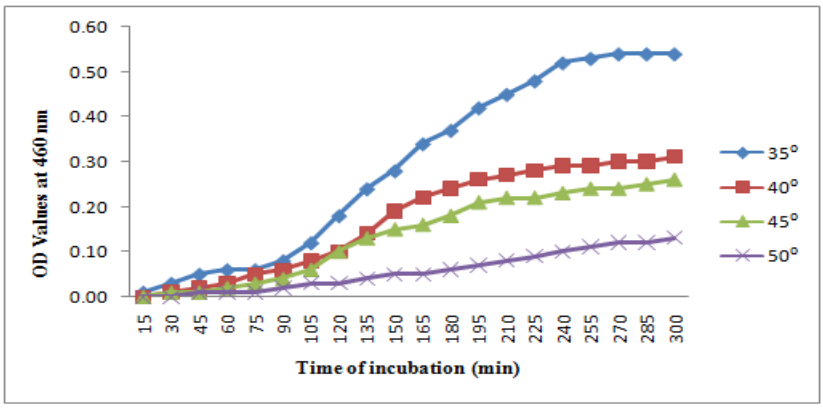

Figure 2: Growth curve of Bacillus methylotrophicus **strain p11**at different temperature

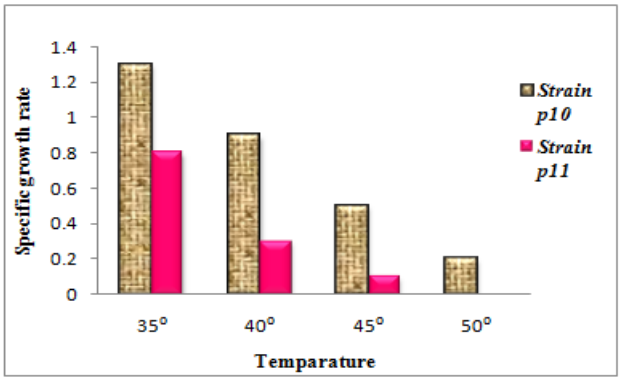

Figure 3: Specific growth rate of Bacillus methylotrophicus **strain p10 & p11**at different temperature regime.

IV. DISCUSSION

Coal mine spoil overburden represents a physically disturbed habitat for the existence of soil organism [12,[13],[14], due to internal high temperature profile [15],[16],and low pH [17],[18]. In spite of such extremities, the coal mine spoil is not a microbiologically sterile habitat and often harbours specific group of thermoacid tolerant, chemolithotrophic and heterotrophic bacteria [19],[20]. Our earlier study revealed Gram positive bacteria to be in a major proportion of total colony forming units of bacterial population in the coal mine soil [6]. There have been also reports about the prevalence of Gram positive bacteria from coal mine soils of different geographical regions [4],[[5]. Similarly in the present study two strains of Bacillus methylotrophicus have been isolated and both of them were found to be Gram positive bacteria.Relatively greater tolerance of Gram positive bacteria to different extremities of habitat is usually explained on the basis of their external lipopolysaccharide layer over and above the cell wall [21].

4.1. Growth Kinetics

The increase in the cell size and cell mass during the development of an organism is termed as growth. Growth analysis of both P10 and P11 strains revealed that both strains were mesothermophilic (35ºC to 50ºC). Perfect sigmoid growth curve was obtained for the strain P10 whereas the growth curve with very long log/ exponential phase was obtained for the strain P11 at 35ºC. The optimum growth of both strains was observed at 35ºC. At other temperatures P10 strain did not reach even the exponential phase of the growth but the growth was slowly increasing with time. At 35ºC, lag phase lasted for about one hour, the exponential phase lasted for about two hours and after three hours only the stationary phase was observed in P10 strain. It was interesting to note that the exponential phase for P11 strain at 35ºC was very long and it lasted for about two hours and forty five minutes. Both the strains were found to be surviving at the higher temperatures like 50°C but the rate of growth was minimum. So the upper threshold temperature must be higher than 50°C. This finding is in line with the report of McMeekin et al.,[22] who reported the temperature dependence of the growth of themophilic bacteria Bacillus sp. and found that minimum temperature for the growth in a submerged culture is close to 20°C, optimum at 51°C, and the upper threshold limit for surviving is 62°C. For the growth of Thermus aquaticus, it was reported that the lower threshold limit of temperature is 20°C, optimum can be found between 70–72°C, and the upper limit of surviving is near 79°C [23]. Based on recent data published by Barakat and Harris [24] and Augustin et al. [25], Adriane Nunes de Souza et al.[26], Munusamy Madhaiyan et al. [27], K.Melzoch et al. [28]reported that the rate of the colony growth of all the thermophilic bacteria near the optimal temperature was practically the same.

The growth pattern of these isolates over a temperature range starting from 35ºC to 50ºC, as observed in the study depicts their adoptability for the higher temperature tolerance, confirming the observation of Hallberg and Lindstrom [29] and Zhou [30]. Analysis of different growth phases with respect to different temperature regime indicated the optimal growth of the isolates at 35ºC and increase in the temperature above 35ºC affected mostly these exponential growth phase resulting relatively early initiation of stationary phase. Such observation clearly reveals about their basic mesophilic character with adoptability for the higher temperature tolerance. Growth pattern of the Gram positive strain p11 at higher temperature of 50ºC showed total absence of the exponential phase resulting the specific growth rate to be zero. This reflects the poor thermal adoptability of the strain in comparison to the other. However, both the isolates showed maximum value of specific growth rate at 35ºC and the rate declined with increase in the temperature which very conclusively points out their mesophilic character.

Previous workers have demonstrated the existence and nonexistence of gram-positive bacteria in acidic mine waters. Wichlacz and Unz [31] reported that a Bacillus sp., which produced exocellular polysaccharide grew optimally at circumneutral pH, and it was identified as a major member of the slime population. These workers suggested that the predominant gram positive bacteria of the streamers may exist in microzones, with pH values much closer to neutrality than those of the acidic bulk water. The lesser diversity of bacteria in the coal mine soil used in this work can be explained on the basis of acidic nature of soil and a greater permeability of the cell wall to toxic hydro anion as reported by J.H.Tuttle et al [32]. A.P.Harrison,Jr.[33] described that hydrogen ion alone may not be totally responsible for adverse effects on gram-positive bacteria in acidic environments since certain Bacillus spp., e.g., B. coagulans and B. acidocaldarius require distinctly acid conditions for growth.

V. CONCLUSION

The characterization of the small fraction of microbes that has been cultivated provides only a glimpse of their potential physiological capacity and influence on soil ecosystems. During this study, two bacterial isolates, i.e. Bacillus methylotrophicus strains p10 and p11 were isolated from soil. Pure cultures of these bacteria (Bacillus methylotrophicus strain p10 & p11) used for the growth kinetics study. The growth pattern of these isolates over a temperature range starting from 35ºC to 50ºC was observed in and the growth pattern of the strain p11 at higher temperature of 50ºC showed total absence of the exponential phase resulting the specific growth rate to be zero. This reflects the poor thermal adoptability of the strain in comparison to the other. However, both the isolates showed maximum value of specific growth rate at 35ºC and the rate declined with increase in the temperature which very conclusively points out their mesothermophilic character. Further studies on the physiological viability and cellular enzymatic characterization of these two isolates with respect to the higher temperature regime may throw more light on their bioprospecting ability in the context of their industrial application.

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