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Study on the mechanism of *Azotobacter chrococum* MTCC 446 after degradation of phenol and cyanide

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ABSTRACT

Cyanide and phenol are emitted generally from various industries in a large scale as toxic pollutants for living organisms. In spite of toxicity, many micro-organisms take these elements as a carbon or nitrogen sources for their metabolic activities and produces other compounds which are less harmful for environment. Biodegradation is one of the safest methods for degradation of such type of carcinogenic pollutants. In this paper, Azotobacter chrococum was used for biodegradation of cyanide and phenol and their mechanism during the degradation has been observed. Azotobacter chrococum degraded cyanide and formed methane as an end product which indicated that it followed reductive pathway among all of five pathways. Gas Chromatography (Model MICHRO-9100) detected methane produced during cyanide degradation at the retention time 0.26 min. On the other hand, during the degradation of phenol, conversion of catechol to 2-hydroxymuconic semialdehyde catalyzed by catechol 2,3-dioxygenase and also absence of cis, cis-muconic acid indicated that Azotobacter chrococum metabolize phenol via meta-pathway.

Keywords: Azotobacter chrococum, Phenol degradation, Cyanide degradation, methane, ammonia, meta-cleavage pathway

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INTRODUCTION

Cyanide and phenol both are poisonous, carcinogenic and toxic environmental pollutants. Cyanide is highly toxic to human beings because it interrupts in the function of cytochrome oxidase that can create a blockage in respiratory system and ultimately causes death [3]. Phenol is also poisonous and its exposure to human beings causes highly irritation to the skin, eyes. Usually concentration of phenol can be tolerated1.5 g/l but sometimes in polluted waters this may be increased to 4.5 g /l [2]. The sources of these pollutants are commonly various industries. Phenol is discharged generally oil refineries, paper processing plants, resin production, and coal liquefaction [12] and Metal-plating, pharmaceutical industries are the most common sources of cyanide. It has been reported that the limitation of cyanide concentration in the wastewater should be < 0.1 g / l [10].

Several methods such as alkaline chlorination, ozonization, wet-air oxidation, adsorption, precipitation, volatilization etc have been applied for remediation of these toxic pollutants. But these methods are very expensive as well as also causes health hazard due to use of chlorine and other chemical reagent. Despite toxicity of these pollutants, several microorganisms utilize these as a sole source of carbon or nitrogen. Biodegradation of the toxic pollutants is cheaper than the other methods of remediation and also environmentally safe.

For degradation of cyanide there are generally five pathways [1,4,11,14]. As end products microbes finally produce ammonia, formic acid, formate, formamide, carbon-di-oxide etc. The pathways are Hydrolytic pathway, Oxidative pathway, Reductive pathway, Substitution/transfer pathway, Syntheses pathway. Phenol is degraded to catechol via ortho or meta-cleavage pathway. If the ring fission is catalysed by ortho enzyme catechol 1,2-dioxygenase the product would be cis,cis-muconic acid and if the ring fission is catalysed by meta enzyme 2-3-dioxygenase then 2-hydro cis muconic semialdehyde would be formed [8].

MATERIALS AND METHODS

Plating of Bacteria

Microbial strain of *Azotobacter chrococum* (MTCC 446) was purchased from MTCC, Chandigarh, India. Nutrient Broth and Nutrient agar was obtained from HiMedia Laboratories Pvt. L.T.D. Mumbai. To prepare 25 ml nutrient agar solution 1 g of nutrient agar was added in 30 ml of distilled water. Then the solution was autoclaved at 121 ° C for 20 min. After cooling the solution, it was poured into the petridish which was kept at room temperature for overnight to solidify the media. Microorganism was scooped from the nutrient broth through sterilized wire loop and was streaked in the petridish incubated at 30° C for 24 h. After seeing the growth of the colonies of microorganism it was preserved at 4 °C (figure 1).

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Preparation of Minimal Media and Cell Free Extract

For preparation of minimal media following reagents were added in 800 ml solution. The pH of the solution was 7.(Liu et al.[6]). Chemicals used to prepare minimal medium: Na2HPO4 8.8g/l, KH2PO4 1.2g/l, NaCl 5g/l, NH4Cl 1 g/l, MgSO4, 7 H2O 0.25 g/l, Glucose(w/v) % 2g/l. Media was autoclaved at 120^{o} C. The media was allowed to cool at room temperature. The solution was equally distributed into two serum bottles. In one bottle potassium cyanide was added 0.5g in order to make concentration of potassium cyanide 50 mg/l and in the other bottle 18.7 µl phenol was added for making concentration 50mg/l. Again each solution was divided into two parts. The two microbial strains were inoculated into each flask in laminar hood. Subsequently, the bottles were incubated at the shaker for 48 hours at 260 rpm. For preparation of cell free extract the incubated culture medium of both bacteria was centrifuged for 10 min at 8000 rpm. The clear solution was preserved for formamide and ammonia analysis.

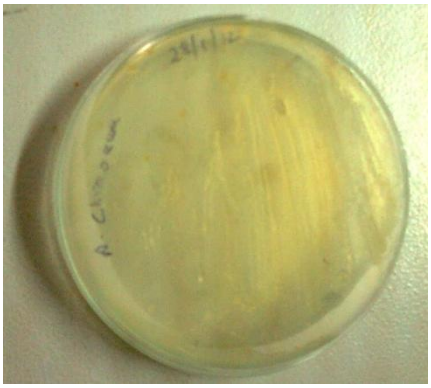

Figure 1: plating of bacteria

Cyanide Degradation

When cyanide is degraded by different micro-organisms, It can be converted to one or more than of the following as end products, i.e: methane, formamide, formate, ammonia, carbon-di-oxide etc.

Analysis of Methane

Because of nitrogenase activity of some microbes [7] in the presence of cyanide, methane can be produced. After incubation for 48 hours, the gas phase (1 ml) was collected through syringe from the serum bottles and methane was analyzed using a NUCON-5700 Gas chromatography (GC), flame ionization detector with a Porapak N 80/100 column.

Analysis of Formamide

For formamide analysis following method was used where basically formamide converts to ferric hydroxamate [4]. 0.2 ml of the sample (cell free extract) was incubated with the mixture (0.4ml) containing 3.5 N of NaOH and 2.3 M of hydroxylamine hydrochloride (1:1)

for 10 min at 65 ^{o} C. After that, 0.2 ml of 4 N HCl and 0.2 ml of 1.23 M $FeCl_3$ were added. Then the absorbance at 540 wave-length was read for 5 min later after the addition of $FeCl_3$.

Analysis of Ammonia

For analysis of ammonia colorimetric method was used. 4 drops of Nessler's reagent were placed into two tubes by using sterile disposable pipette. After adding Nessler's reagent, two microbial cell extracts were dropped into each tube. There are four option to understand the presence of ammonia. Clear = no reaction, Yellow = some reaction, Brown = more reaction, Brown + precipitate = greatest reaction.

Phenol Degradation

The bacterial solution grown in the presence of phenol was centrifuged for 10 min at 8000 rpm and washed 0.033M Tris-HCl buffer (Ph 7.5) to remove the salts and again it resuspended in Tris-HCl buffer. Cells were disrupted by using the tip sonicator for 15 min**.** In order to remove the phenolics Polyvinylpolyprrolidone (PVP) was added. Then the samples were centrifuged at 10000 rpm for 30 min. The clear supernatant solution can be called as cell free extract. It was preserved in ice and further analyzed for dioxygenase activity.

Determination of Ortho-cleavage Pathway

Formation of cis,cis-muconic acid indicates the activity of catechol 1,2 dioxygenase. The following reagents were added in a test tube: 4ml of 50mM Tris HCl buffer (pH7.5); 1.4 ml distilled water, 0.1 ml 200 mM 2-mercaptoethanol; 0.2 ml cell-free extract. The solution was mixed by inversion and 0.2 ml catechol (5µM) was then added and the contents were mixed again.The increase in the absorbance at 260 nm over a period of 5 min were followed in a UV-shimadzu spectrophotometer [9].Increase in absorbance confirmed the formation of cis,cis muconic acid.

Determination of Meta-cleavage Pathway

Catechol 2, 3-dioxygenase activity on the degradation of phenol was measured by the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol. The following reagents were added to a test tube: 4 ml 50 mM Tris-HCl buffer (pH 7.5); 1.2 ml distilled water; 0.4 ml cell free extract. The solution was mixed by inversion and 0.4 ml catechol (50 mM) was added and mixed with the contents. The production of 2-hydroxymuconic semialdehyde was followed by increase in absorbance at 375 nm over a period of 5 min. [13].

RESULTS AND DISSCUSSION

After inoculation of both bacteria into minimal media in the presence of phenol and cyanide the growth of bacteria was observed through increasing the turbidity of the culture medium.

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Analysis of methane by GC:

To determine the cyanide metabolic pathway for *Azotobacter chrococum* 446 methane analysis was performed by GC. To know the retention time of pure methane 1 ml of pure methane was injected in the injection port of GC. After 0.25 min a peak has been appeared. After that 1 ml of each gaseous sample from the incubated serum bottles containing the strain was injected.In that case a peak appeared at the retention time 0.26 min .This results proved that methane was produced in the serum bottles.(figure 2 and 3).

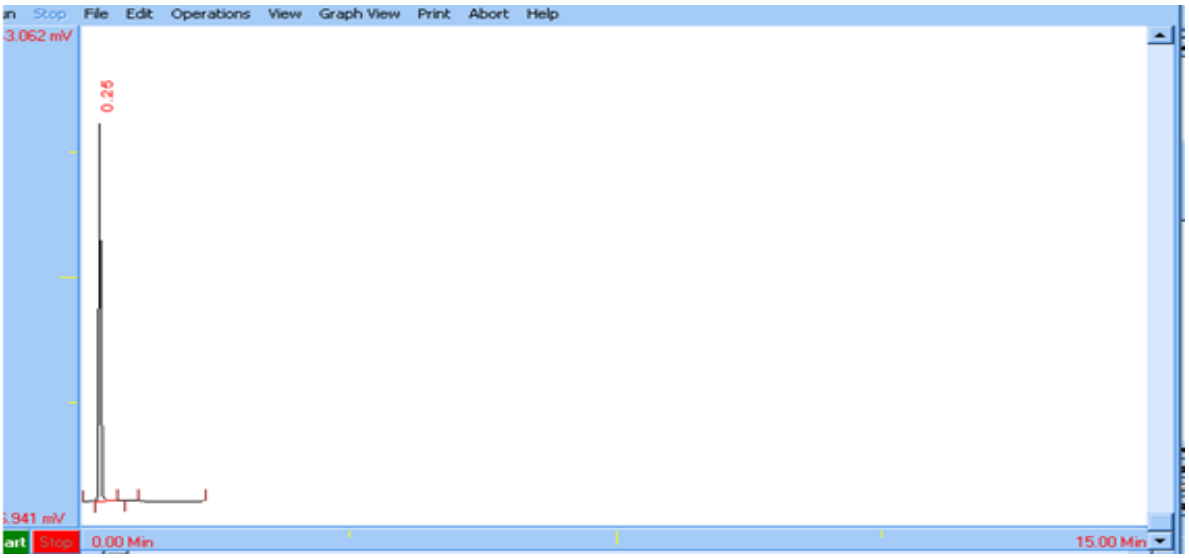

Figure 2: GC-curve for pure methane

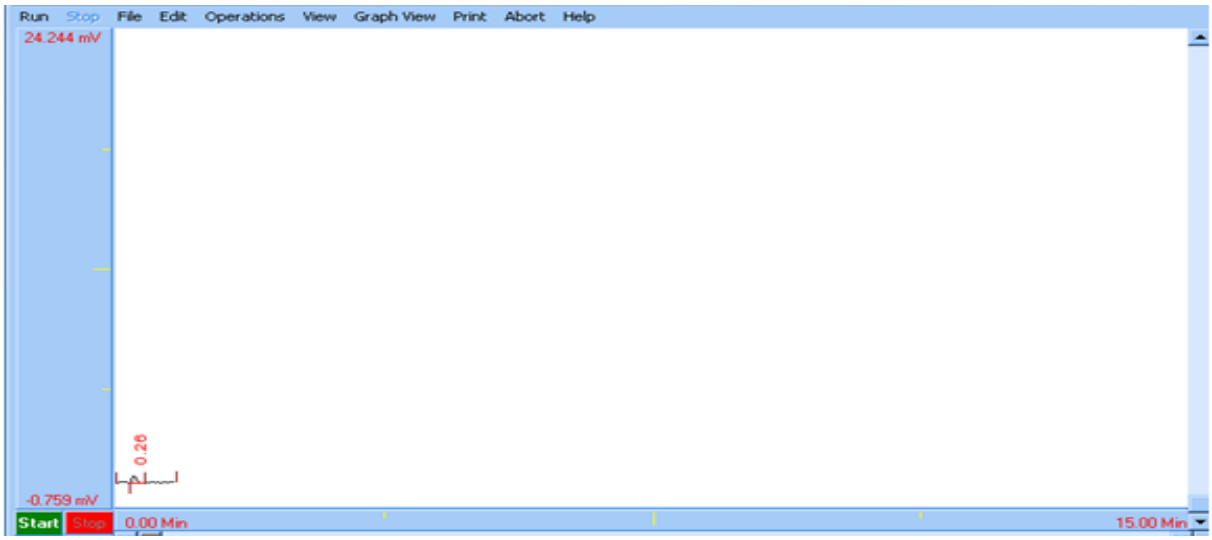

Figure 3: GC curve for gas sample collected after degradation of cyanide by *Azotobacter chrococum* 446

For ammonia analysis colorimetric method was used. To prepare cell free extract culture medium was centrifuged for 15 min at 8000 rpm. The supernatant solution was used for

ammonia analysis. After addition of four drops of nessler's reagent through sterilized pipette into the sample of both strains, brown precipitated was formed. So the strain formed ammonia after degradation of cyanide.

From this analysis, it is evaluated that *Azotobacter chrococum* 446 biotransformed cyanide into methane and ammonia. Here by it is confirmed that nitrogenase enzyme degrades cyanide under anaerobic condition. So, the pathway followed by *Azotobacter chrococum* 446 was reductive pathway. For this case, formamide has not been detected. Thus, cyanide hydratase was not involved in the biodegradation process.

Determination Phenol degradation Pathway

After addition of catechol (5µM), there was a decrease in the absorbance at 260 nm after 5 min for *Azotobacter chrococum* species (Table 1). Decreasing in absorbance at 260nm indicates the absence of cis,cis muconic acid catalyzed by Catechol 1, 2 dioxygenase. So it is assumed that *Azotobactor chrococcum* metabolize phenol via meta pathway.

Time (min)	Absorbance
0	.003
5	.001

Table 1: Spectrophotometric reading at 260 nm wavelength for *Azotobacter chrococum*

Time (min)	Absorbance
0	.001
5	.007

Table 2: Spectrophotometric reading at 375 nm wavelength for *Azotobacter chrococum*

Catechol 2,3-dioxygenase activity on the degradation of phenol was measured by the formation of 2-hydroxymuconic semialdehyde, the *meta*- cleavage product of catechol. For determination of meta-pathway ,4 ml 50 mM Tris-HCl buffer (pH 7.5); 1.2 ml distilled water; 0.4 ml cell free extract and 0.4 ml catechol (50 mM) were added and mixed with the contents. Absorbance at 375 nm over a period of 5 min was followed. There was an increasing in absorbance at 375 nm for *A.chrococum* (Table 2). So 2-hydroxymuconic semialdehyde was produced which concluded that *Azotobacter chrococum* metabolize phenol via meta pathway.

CONCLUSION

Azotobacter chrococum 446 was purchased from MTCC, Chandigarh, India. The present study shows that strain was able to grow in the presence of toxic pollutants (cyanide and phenol) and also bio-transformed into different products. *Azotobacter chrococum* 446 degraded cyanide and formed methane and ammonia which was an anaerobic process. It is concluded that *Azotobacter chrococum* 446 degrades cyanide via reductive pathway. *Azotobacter chrococum* 446 degraded phenol by catechol 2,3 dioxygenase activity and formed

2 –hydroxymuconic semialdehyde. In summary it can be suggested that *Azotobacter chrococum* 446 degraded cyanide via meta-pathway.

This study presents us the mechanism of cyanide and phenol biotransformation by *A. chrococum*.This study will be very helpful for biotreatment of toxic pollutants(cyanide and phenol) containing wastewater.

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