



**A study on 3-mercaptopyruvate sulphurtransferase (3-MST)
 produced under submerged fermentation
 by *Pseudomonas putida* KT12440 isolated from an industrial effluent
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Abstract

Effluents from an Iron and Steel company were collected, the cyanide level was determined and screening for cyanide degrading bacterial isolates on modified Bushnell Haas agar media was done. The amount of cyanide present in the industrial effluent was 1.06 mg/L. The 16S rRNA gene sequencing identified *Pseudomonas putida* KT12440 as the isolate with highest activity for 3-MST production. The cell growth was optimal at 30 h incubation time and a decrease in cell growth was observed with the increase of incubation time after the optimum. The highest 3-MST production was observed when mannitol was used as the sole carbon source while fructose is the least source of carbon. Casein showed the highest production for 3-MST when used as an alternative nitrogen source while NH₄NO₃ was the least. The optimal pH for 3-MST production was 9.0 and a sharp decrease in the enzyme production was observed thereafter. The incubation temperature for the production of 3-MST in the culture medium peaked at 30°C and a sharp decline in temperature was observed afterward. The bacterial isolate screened in this study showed degradation potential that can be harnessed to remediate effluents containing cyanide.

Keywords: Cyanide, Industrial Effluent, *Pseudomonas putida* KT12440, 3-MST

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Introduction

Cyanide is a carbon-nitrogen radical that can be found in a wide variety of organic and inorganic compounds and minute amounts of cyanide in form of vitamin B12 (cyanocobalamin) are necessary requirement in human diet. However, cyanide, in some forms (HCN), is a very powerful and fast acting toxin. Its toxicity to living cells has been observed (Solomonson, 1981; Knowles, 1988). It is a potentially deadly chemical which prevents or inhibits cellular respiration and inactivation of cytochrome oxidase, hence death of body cells. Cyanide has been reported to be readily adsorbed through different parts of the body (Banerjee et al., 2002).

It has been observed that the most significant and major sources of cyanide releases to water are discharges from metal finishing or mining industries/processes, iron and steel mill plants, publicly owned wastewater treatment facilities and organic chemical industries (Ubalua, 2010). Numerous accidental spills of sodium cyanide or potassium cyanide into rivers and streams have been reported to be the cause in massive killing of fishes, amphibians, aquatic insects, and aquatic vegetation. The hazard of cyanide level above the acceptable limit to fish, wildlife, livestock and man have been well documented (Solomonson, 1981; Sepúlveda et al., 2010).

A number of industrial effluents contain cyanide at a concentration exceeding 100 mg/L (Watanabe et al., 1998; Gurbuz et al., 2009). The discharge of large amounts of cyanide compounds in the environment imposes a serious threat to ecosystem sustainability (Sepúlveda et al., 2010; Das & Santra, 2011). There are various treatment processes used for the detoxification of cyanide but the biological detoxification of cyanide has been reported to be an attractive alternative to chemical detoxification (Dash, 2009; Kumar et al., 2013). Biodegradation of free cyanide from industrial waste waters has been proven as a viable and robust method for treatment of wastewaters containing cyanide.

Biological detoxification methods may be both cheaper and more environmentally acceptable than chemical methods. Some microorganisms have the ability to degrade cyanide, due to the presence of a detoxifying enzyme such as 3-Mercaptopyruvate sulphurtransferase that converts cyanide to thiocyanate. In addition, enzymes produced by microorganisms are more predictable and controllable and the raw materials for production are readily available with ease of production (Murad & Azzaz, 2011). Bacteria compared to other microorganisms have been shown to possess high tolerance and degradation ability for cyanide (Kumar et al., 2013). Hence, this study aims to characterize the production of 3-mercaptopyruvate sulfurtransferase which converts cyanide to thiocyanate, a less toxic compound by bacteria isolated from industrial effluent under submerged fermentation.

Materials and Methods

Materials

Nutrient agar, Gram's iodine, potassium cyanide, sodium thiosulphate, peptone, yeast extract, formaldehyde, ferric nitrite, nitric acid, citric acid, sodium citrate, ammonium sulphate (enzyme grade), potassium chloride and sodium chloride were obtained from BDH Chemical Limited, Poole, England. Disodium hydrogen phosphate

and monosodium dihydrogen phosphate were products of Kermel Reagent Company Limited, Tianjin, China. Other chemicals, solvents and media ingredients used for the experimental purpose were of analytical grade and were procured from reputed chemical firms.

Collection of Samples

The waste water effluents were collected from Ile-Ife Iron and Steel Company using a 200 ml sterile bottle by submerging the bottle to a depth of 20 cm. The samples were labeled, transported to the laboratory in ice packs and subsequently analysed within 30 minutes to 1 hour of collection.

Determination of Cyanide Level in Water

The level of cyanide in the effluent was determined using the alkaline titration method as described (AOAC, 1990). Ten 20 ml of the effluent was aliquoted in 800 kjeldahl flask, Calcium and 200 ml water were added and allowed to stand for 2 - 4 h. In the steam distillation, 150 160 ml of the distillate was collected in NaOH solution, and diluted to definite volume. To about 100 ml of the distillate, 8 ml 6 N NH_4OH and 2 ml 5% KI solution was added and titrated with 0.02N AgNO_3 , using microburette.

Screening of Cyanide Degrading Bacteria

A loopful of the effluent stock sample were streaked on modified Bushnell Haas agar plates containing 0.3% KCN in triplicates under aseptic condition and incubated inverted at 25°C for 96 h to screen for cyanide degrading bacteria (Parmar et al., 2012).

Isolation of Cyanide Degrading Bacteria

Colonies observed on the modified Bushnell Haas agar plates were subcultured for pure isolates and stored in nutrient agar slants under refrigeration at 4°C.

Activity of Bacterial isolates for 3-MST Production

The bacterial isolates cultured were screened for highest activity for 3-MST production under submerged conditions. The isolates were standardized to 0.5 McFarland and 1 ml each of the inoculum were introduced into conical flasks containing sterile KCN at concentrations of 30 mg per 100 ml Bushnell Haas agar medium. The cultures were incubated at 37°C for 48 h in an orbital shaker incubator at 170 rpm. The cultures were centrifuged at 12 000 rpm for 20 min to separate the cell from supernatant. The crude supernatants were used to evaluate 3-MST activity (Taniguchi & Kimura, 1974).

Phenotypic and Molecular Identification (16S rRNA gene sequencing) of the Bacterial Isolates

The identification of 3-MST producing bacterial isolate was performed using traditional microbiological and molecular techniques. The presumptive identity of the bacterial isolate with the highest 3-MST activity was determined with reference to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Extraction of DNA was done using Cetyl Trimethyl Ammonium Bromide (CTAB) method as described (Trindade et al., 2007). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primer derived from the 16S Ribosomal DNA sequence of *E. coli*. Forward primer 27 forward: AGAGTTTGATCMTGGCTCAG and reverse primer 1525 reverse: AAGGAGGTGWTCCARCC were used for the polymerase chain reaction (PCR). PCR amplification was performed in 25 µl containing 4 µl of the DNA solution, 0.4 µl of 10 mM dNTPs, 2 µl of 25 mM MgCl₂, 1 µl of 10 µmol each of primer, 0.24 µl of Taq polymerase (1 U/µL) (Promega USA) and the 5 µl of 5× PCR buffer. Sterile DNase free water was added to make a volume of 25 µl. Thirty-five PCR cycles were conducted in automated thermal cycler. The thermal conditions were as follows: denaturation at 94°C for 1 min (3 min for the first cycle), annealing at 56°C for 1 min and extension at 72°C for 1 min.

This was followed by one cycle of final extension at 72°C for 7 min. The PCR amplicon was analyzed in 1.5% agarose gel electrophoresis with amplification product at 1500 bp (Universal Bacterial PCR Fact Sheet, 2004). The amplicons were purified with the addition of 70% ethanol and centrifugation at 9000 rpm before nucleotide sequencing, using ABI 3130xL Genetic Analyzer (Applied Biosystems, California, USA). Sequence similarity search of the GenBank database was done using the NCBI Basic Alignment Search Tool (BLAST) program.

3-Mercaptopyruvate sulphurtransferase Assay

3-Mercaptopyruvate sulphurtransferase (3-MST) activity was measured according to the modified method of Taniguchi & Kimura (1974) as described by Agboola et al. (2006) using mercaptoethanol as substrate. The reaction mixture in a final volume of 1 ml contained 0.38 M of TrisHCl buffer, pH 7.8, 0.5 M potassium cyanide, 0.25 M mercaptoethanol and 100 µl of enzyme solution. The mixture was incubated for 15 min at room temperature (25 °C) and the reaction was stopped by adding 1.5 ml of 38% formaldehyde, followed by the addition of 1.5 ml of sorbo reagent. Absorbance was also measured at 460 nm. The unit of enzyme activity was defined as micromoles mercaptocyanate formed per minute at 37°C and pH 7.8. Protein concentration was estimated using Bradford method (1976) bovine serum albumin (BSA) was used as standard.

Effect of Period of Incubation Time on Enzyme Production

The inoculated media were incubated at 37°C for 48 h under agitation at 170 rpm and subjected to various incubation time. At intervals of 2 h, 5 ml of samples were collected aseptically for a period of 48 h, the optical density of each sample was checked at 590 nm using a colorimeter and recorded as the cell optical density. The samples each at the different time intervals were further centrifuged at 12000 rpm and a cell free

supernatant was obtained. The supernatant which served as the crude was checked for 3-MST activity at 460 nm and the protein at 595 nm.

Effect of Carbon Sources on 3-MST Production

Different carbon sources such as glucose, sucrose, maltose, mannitol, dextrose and lactose at a concentration of 1% were used as alternative replacements for potassium cyanide in the basal medium and inoculated with 1 ml of standard bacterial suspension. The culture flasks were incubated at 37°C for 30 h on a rotary orbital shaker at 170 rpm.

Effect of Nitrogen Sources on 3-MST Production

Peptone was replaced with various nitrogen compounds such as ammonium nitrate, ammonium sulphate, potassium nitrate, ammonium chloride, sodium nitrate, glycine, casein, phenylalanine, peptone and urea. The nitrogen compounds were added at 1% concentration in each conical flask containing 100 ml aliquot of the sterile modified medium of appropriate nitrogen compound and inoculated with 1 ml of standard bacterial suspension. The culture flasks were incubated at 37°C for 30 h on a rotary orbital shaker at 170 rpm.

Effect of Hydrogen Ion Concentration (pH) on 3-MST Production

The pH of the growth medium ranged from 4-10 at 1.0 interval variation using different buffers whose buffering capacity is within the required pH. The initial pH of the media was adjusted to different pH and inoculated with 1 ml standard bacteria suspension and incubated at 37°C for 30 h on a rotary shaker at 170 rpm.

Effect of Temperature on 3-MST Production

Conical flasks (250 ml) each containing 100 ml of growth medium for 3-MST production was inoculated with 1 ml of standardized bacterial suspension. The flasks were incubated at temperature ranging from room temperature (25 ± 2), 30, 35, 40, 45°C and incubation time was 48 h. The crude enzyme was collected and assayed for 3-MST activity and protein concentration.

Results

The amount of cyanide present in the industrial effluent was 1.06 mg/L. The 16S rRNA gene sequencing identified, *Pseudomonas putida* KT12440 (Plate 1) as the isolate with highest activity for 3-MST production. The cell growth was optimal at 30 h incubation time and a decrease in cell growth was observed with the increase of incubation time after the optimum (Fig. 1).

The highest 3-MST production was observed when mannitol was used as the sole carbon source while fructose the least source of carbon as shown (Fig.2). Casein showed the highest production for 3-MST when used as an alternative nitrogen source while NH_4NO_3 was the least (Fig. 3). As shown in Fig. 4, the optimal pH for 3-MST production was 9.0 and a sharp decrease in the enzyme production was observed at pH after the optimum.

The incubation temperature for the production of 3-MST in the culture medium peaked at 30°C and a sharp decline in temperature was observed afterward (Fig. 5).

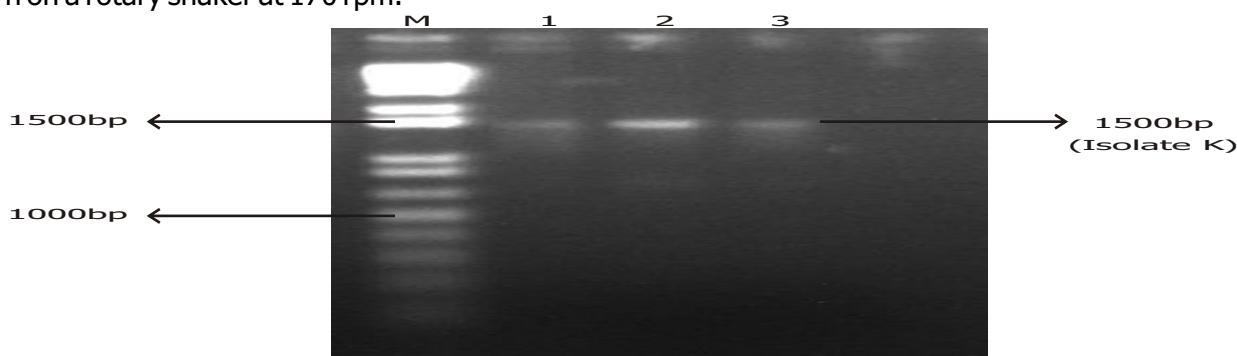


Plate 1: Agarose Gel Showing 1500bp of the 16S RNA Amplicon Band of the Isolate. Lane M= Molecular weight marker, Lane 1 - 3 Isolate K

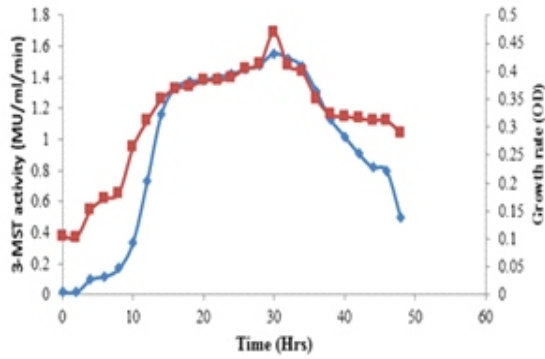


Figure 1: Effect of Incubation Time on 3-MST Production and Cell Growth
 Legend: Growth rate (OD) (blue line), 3-MST activity (red line)

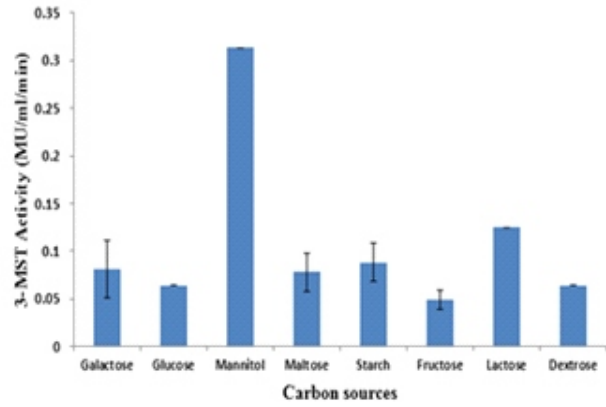


Figure 2: Effect of Carbon Sources on 3-MST Production by *Pseudomonas putida* KT 12440

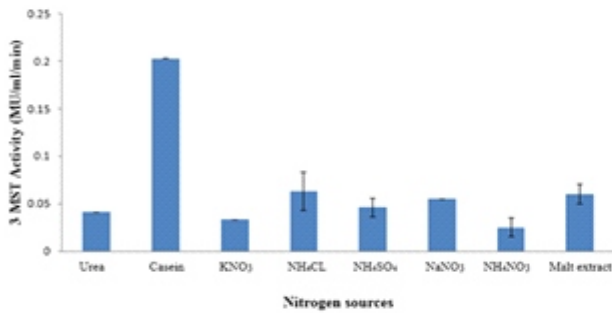


Figure 3: Effect of Nitrogen Sources on 3-MST Production by *Pseudomonas putida* KT12440

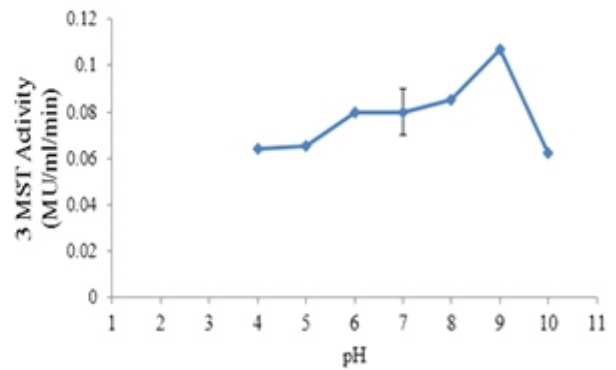


Figure 4: Effect of pH on 3-MST Production by *Pseudomonas putida* KT12440

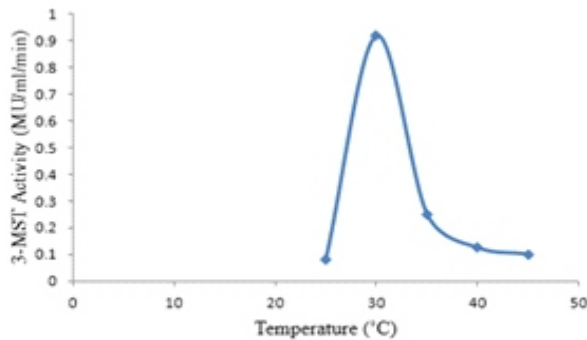


Figure 5: Effect of temperature on 3-MST production by *Pseudomonas putida* KT12440

Discussion

The growth and survival of cyanide- detoxifying bacteria can be said to be due to the ability of the organism to breakdown cyanide into several other compounds which are eco-friendly (Dash et al., 2009). It was observed in this study that the amount of cyanide present in the effluent was 1.06 mg/L, which was 5.3 times higher than the recommended 0.2 mg/L acceptable limit of

cyanide in industrial effluents (USEPA, 1989). It has been posited that cyanide level in industrial effluents discharged into the environment that is above the acceptable limit can pose serious adverse effect on human and environment health. Studies have reported the isolation of microorganism involved in cyanide metabolism (Knowles, 1988; Kumar et al., 2013).

In this study, the cyanide detoxifying microorganism was identified as *Pseudomonas putida* KT2440. In *Pseudomonas putida* KT2440, 3-MST production was evident in the culture supernatant after 2 - 4 hours of incubation. The peak of the 3-MST production was at 30th hour with gradual decline. The decline in the 3-MST production may be due to exhaustion of nutrients or accumulation of other products or metabolites which are inhibitory to the growth of the bacterium and 3-MST production. This has been noted by Goyal et al., (2005) and Prakash et al., (2009) who stated that inhibition of enzyme production may occur as a result of catabolic repression by metabolizable monosaccharides such as glucose, increase in concentration of protease and rapid change in pH with the aim of conserving energy by bacteria.

The sources of carbon available to 3-MST producing bacteria was found to be mannitol with the enzyme activity of 0.313 MU/ml/min. Mannitol has been reported to be one of the most abundant energy and carbon sources in nature produced by a diverse organism, including bacteria, yeasts, fungi, algae, lichens and many plants (Song and Vieille, 2009). A wide range of nitrogenous compounds, either organic or inorganic has been shown to affect the productivity of 3-MST. The nature and relative concentration of different complex nitrogenous sources in the growth medium are both important in the synthesis of 3-MST. The effect of nitrogen sources on 3-MST in this study showed the enzyme was produced maximally with casein at 0.203 MU/ml/min followed by peptone with 0.081 MU/ml/min. This may be due to the fact that casein with its relatively little tertiary structure can readily be made available to the bacterium for metabolism during growth and enzyme production as opposed to other nitrogen sources.

Microbial growths are influenced by pH and temperature in the production of enzyme. The optimum pH for the production of *Pseudomonas putida* KT2440 3-MST was 9.0 with enzyme activity of 0.107 MU/ml/min. Most environments are between pH 5.0 and 9.0 with optima between these values. Panos and Bellini (1999)

reported pH of 9.0 for optimum microbial cyanide degradation. Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this usually varies from one organism to another (Banargee & Bhattacharya, 1992; Kumar & Takagi, 1999). Panos and Bellini (1999) reported that cyanide microbial activities increased as the temperature rose to 37°C and an optimum was observed at 30°C. But, in this study, *Pseudomonas putida* KT2440 was found to grow optimally at 25-30°C.

The production of 3-mercaptopyruvate sulfurtransferase by *Pseudomonas* spp. is often dependent on the growth of the bacterium in the appropriate media composition. The bacterial isolate screened in this study showed the potentials for degradative activities that can be harnessed to remediate cyanide wastes.

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