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Original Research Paper

BIODEGRADATION OF SALICYLIC ACID FROM SOIL ISOLATES

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ABSTRACT

In this present work of study, 16 different bacterial strains were isolated from various environments. The study showed that 8 isolates were capable of degrading salicylic acid. The most efficient degrader was isolate D1. The BHM media was used for the salicylic acid degradation. The ability of isolate to degrade salicylic acid was tested by scanning the spectrum with UV-Visible spectrophotometer. The isolate D1 showed maximum degradation of salicylic acid in 48 hrs of incubation. The maximum degradation of salicylic acid was observed at pH-7 and temperature at 25ºC and 37ºC. The organism showed efficient degradation when yeast extract was used as nitrogen source. Further increase in the salicylic acid concentration slowed down the rate of degradation.

Keywords: Salicylic acid, Bioremediation, Polyaromatic hydrocarbon, UV-Visible spectrophotometer.

INTRODUCTION

The word "Bioremediation" states, bio: the live component and remediation: the treatment of the contaminant. The pollutant such as organic or inorganic are removed by the natural strain of microorganisms. Bioremediation is the process where wastes are degraded into harmless product by enzymatic action of microorganisms. The microbes use organic substances as a source of carbon and energy. Polyaromatic hydrocarbons (PAHs) composed of two or more fused aromatic rings arranged in linear or cluster. The aromatic ring is broken down to PAH metabolites or carbon dioxide by oxidation. The salicin containing both aromatic component and sugar known as salicylic acid. Salicylic acid is a 2-hydroxybenzoic acid (BHA). A drug that has bactericidal and fungicidal properties. A number of analgesic, anti-inflammatory drugs, such as acetylsalicylic acid, are prepared from it. Salicylic acid is a key ingredient in many skin-care products for the treatment of acne, psoriasis, keratosis pilaris, and warts, salicylic acid is used in several shampoos. Salicylic acid is biosynthesized from the amino acid phenylalanine. Sodium salicylate is commercially prepared by the Kolbe-Schmitt reaction. However, salicylic acid can be quite toxic if taken in large doses.

Salicylic acid degradation is carried out by various bacteria. The enzymes responsible for degradation of polyaromatic hydrocarbon are able to degrade salicylic acid. Salicylic acid degradation to catechol and then cleaved via the *meta* or *ortho-pathway*. Salicylic acid can also be oxidized through gentisic acid. In aerobic conditions, oxygenase (monoxygenase and dioxygenase) enzymes is responsible. While in anaerobic bacteria such as sulphate-reducing bacteria is involved for salicylic acid degradation. Colorimetric and spectrophotometric techniques were selected to evaluate the effectiveness of Salicylic acid quantification. Although the Trinder test is rapid and inexpensive to perform, its selectivity is poor. Salicylic acid is an enol of a β-ketocarbonic acid and therefore forms purple complexes with iron salts in solution read on optical density of 525 nm. TLC can be used to assess the course of a

reaction, to assess the purity of a sample and also to identify unknown compounds by comparison with standards. In this experiment, the UV light or iodine vapour is used for the detection of spots.

MATERIALS AND METHODS

Media

All media components and chemicals used in the studies were of high purity and analytical grade. The medium used was Bushnell Hass Media (BHM) containing salicylic acid (500 ppm) .

Sample Collection

Soil sample were collected from the various chemically contaminated sites of Vapi, Gujarat region.

Isolation of Salicylic Acid Degrading Microorganism

Enrichment was carried out by inoculating 1g of soil sample in distilled water. Different dilutions were prepared of soil sample. The dilution were streaked on the BHM agar plate. The BHM plate were incubated for 24 - 48 hrs at 30°C. The isolated colonies were grown on BHM agar plate.

Storage and Maintenance of Pure Culture

The isolated organisms were streaked on BHM agar slants. The slants were incubated at 30°C for 48 hrs. The pure culture is then stored in refrigerator at 4°C and sub cultured periodically.

Degradation Experiment

Inoculum preparation

Inoculum was prepared by transferring preserved culture and growing the cells in 100 ml Erlenmeyer flask containing 50 ml nutrient broth. The flasks were incubated at 30°C for 24 hrs. The freshly grown 24 hrs old culture with 1.0 O.D. at 600 nm is used as Inoculum to inoculate degradation medium BHM broth containing 500 ppm salicylic acid.

Media for degradation

Composition of medium for degradation BHM containing (g/ L); $MgSO_4$ 0.2, $CaCl_2$ 0.02, K_2HPO_4 1.0, KH_2PO_4 1.0, $NaNO_3$ 1.0, $FeCl_3$ 0.5 was sterilized at 121°C, 15 lbs for 15 minutes.

Inoculation of medium for degradation

The sterilized medium was inoculated with 500 ppm salicylic acid and 1% (v/v) of 24 hrs old culture. The inoculated flask was allowed to incubate at 30°C for 48 hrs. The sample was withdrawn at 24 hrs of interval and supernatant was subjected to centrifugation at 5,000 rpm for 20 min and degradation rate was determined.

Estimation of salicylic acid

As the biodegradation is carried out during experiment .The salicylic acid concentration was reduced and this concentration of salicylic acid was estimated by Trinder method, where the acid is quantified in solution in the visible region 530nm (OD 530) by using colorimeter.

Salicylic acid degradation

Degradation experiments were carried out on a shaker at RT, pH 7.0 and 120 rpm. Small amount of this solution was taken in two graduated centrifuge tubes separately and were centrifuged at 8000rpm for 5min. so that all cells get settled down at the bottom. From both graduated centrifuge tubes 1ml of supernatant was taken in two dilution tubes separately and 1ml of 0.02M $FeCl_3$ was added. Tubes were incubated for 5min and 1ml of distilled water was added and taken absorbance at 530 nm, procedure was Repeated for every day until the conc. of salicylic acid become zero

Screening of isolates having degradation activity

Bacteria were selected on the basis of degradation capability using salicylic acid. Bacteria which shows higher degradation activity is selected and further study is carried out using it. The screening of isolates degrading salicylic acid was measured as decrease in optical density using spectrophotometer.

Optimization for cultural conditions for effective degradation

Degradation of salicylic acid was done by promising isolate and carbon, nitrogen source, pH, temperature, medium and incubation period were optimized.

Effect of incubation period

In present study, the effect of incubation period was determined by incubating medium at different incubation periods such as 6, 12, 18, 24, 30, 36, 42, 48 hrs. In Erlenmeyer flask, 100 ml of BHM broth, 500 ppm salicylic acid and 1% inoculum were added and incubated at 30°C. The sample was withdrawn from medium at respective time interval and subjected to centrifugation at 5,000 rpm for 20 min and the supernatant was used for determination of degradation.

Effect of pH

The effect of pH on degradation was studied by incubating BHM medium at different pH such as pH-4, pH-5, pH-6, pH-7, pH-8, pH-9 using 1N NaOH and 1N HCL after sterilization. Each flask inoculated with 1% inoculum incubated at 30°C for 48 hrs and tested for salicylic acid degradation.

Effect of temperature

Effect of varying temperature were 15°C, 20°C, 25°C, 30 °C, 37°C were determined by incubating medium at different temperature. The supernatant was used for determination of salicylic acid.

Effect of carbon source

Various carbon sources were used in order to find out the best one giving high degradation rate. Carbon such as sucrose, glucose, fructose, maltose, lactose, and sucrose were used. Each flask containing 100 ml of medium were inoculated with 500 ppm salicylic acid, 1% inoculum and incubated at 30°C for 48 hrs. At interval of 24 hrs, 5 ml of sample were withdrawn from each flask and centrifuged at 5,000 rpm for 20 min. The supernatant were used to determine degradation of salicylic acid by UV-Visible spectrophotometer at 525 nm. Carbon source giving best degradation rate was optimized.

Effect of nitrogen source

Nitrogen such as peptone, ammonium sulphate, urea, ammonium chloride, yeast extract and beef extract were used. Each flask containing 100 ml of medium were inoculated with 500 ppm salicylic acid, 1% inoculum and incubated at 30°C for 48 hrs. At interval of 24 hrs, 5ml of sample were withdrawn from each flask and centrifuged at rpm 5,000 for 20 min. The supernatant was used to analyze for degradation activity. The best degradation rate obtained by nitrogen source was optimized. The effect of nitrogen source concentration was performed by taking varied concentrations of urea (0.05g, 0.1g, 0.15g, 0.2g, 0.25g and 0.3g) was supplemented to BHM media. Each flask containing 100 ml of medium were inoculated with 500 ppm of salicylic acid, 1% inoculum and incubated at 30°C for 48 hrs. At interval of 24 hrs, 5 ml of sample were withdrawn from each flask and centrifuged at rpm 5,000 for 20 min. The supernatant was used to analyze for degradation activity. The best degradation rate obtained by nitrogen source was optimized. The concentration that gives high degradation rate was optimized.

Effect of different concentrations of salicylic acid on degradation

To check the efficiency of salicylic acid degradation by the bacterial isolate was carried out at different concentration of salicylic acid, 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, and 600 ppm. The flasks containing degradation medium, salicylic acid and 1% inoculum were incubated at 30°C for 48 hrs. Samples were withdrawn at the interval of 24 hrs and subjected to centrifugation at 5,000 rpm. The supernatant was used to determine the degradation of salicylic acid by UV-Visible spectrophotometer at 525 nm and the % degradation was calculated.

RESULTS AND DISCUSSION

Hydrocarbon pollution is known to cause a shift in microbial community with the emergence of new bacterial species having elevated PAH degrading capacity because of assimilation and adaptation of micro-organisms toward organic pollutants. In present study, a total of 16 different bacterial strains were isolated from various environment samples. All 16 isolates were further purified and stored after streaking on BHM agar plate. 8 isolates degrade salicylic acid under submerged condition at 30°C under static condition within 48 hrs of incubation. Bacterial

isolate D1 showed maximum degradation of salicylic acid within 48 hrs of incubation.

Optimization of Cultured Conditions

Effect of incubation period

In present study, the effect of incubation period was determined by incubating production medium at different incubation periods such as 6, 12, 18, 24, 30, 36, 42 and 48 hrs. In Erlenmeyer flask, 100 ml of BHM broth, 500 ppm salicylic acid and 1% inoculum were added and incubated at 30°C. The maximum degradation was shown at 48 hrs.

Effect of pH

The effect of pH on degradation was studied by incubating BHM medium at different pH such as pH-4, pH-5, pH-6, pH-7, pH-8, pH-9 using 1N NaOH and 1N HCL after sterilization. Each flask inoculated with 1% inoculum incubated at 30°C for 48 hrs and tested for salicylic acid degradation. pH is a selective environmental factor affecting microbial diversity and activity, controlling enzyme activity, transport process and nutrient solubility. Lin and Cai (2008) had also reported that pH reduction in the medium was the result of the accumulation of those acidic intermediate products. Hambrick *et al.*,(1990) have also shown that soil bacteria which degrade PAH prefer alkaline condition rather than acidic condition. Pathak *et al.*, (2009) also reported optimum pH for PAH degradation by Pseudomonas sp. is at pH 8. In our study for the degradation of salicylic acid the pH 7 is found to be most effective for the degradation.

Effect of temperature

Effect of varying temperature were 15°C, 20°C, 25°C, 30 °C, 37°C were determined by incubating medium at different temperature. The supernatant was used for determination of salicylic acid. Temperature is one of the important factors affecting the growth and activity of microorganisms. Zhao *et al.*, (2009) and Pathak *et al.*, (2009) also reported optimum temperature for PAH degradation by Pseudomonas sp. is at 37°C. The temperature for degradation of salicylic acid recorded is 25°C and 37°C. Thus the increase or decreased temperature showed no degradation of salicylic acid.

Effect of carbon source

Various carbon sources were used in order to find out the best one giving high degradation rate. Carbon such as sucrose, glucose, fructose, maltose, lactose, and sucrose were used. Each flask containing 100ml of medium were inoculated with 500 ppm salicylic acid, 1% inoculum and incubated at 30°C for 48 hrs. At interval of 24 hrs, 5ml of sample were withdrawn from each flask and centrifuged at 5,000 rpm for 20 min. The supernatant were used to determine degradation of salicylic acid by UV-Vis spectrophotometer at 525 nm. Carbon source giving best degradation rate was optimized. The addition of carbon and nitrogen sources has been increased the growth of microorganisms which resulted in enhanced PAH degradation (Lee *et al.*, 2003). Generally, hydrocarbons are weak sources of nutrients such as nitrogen and phosphorus which are essential building blocks of structural macromolecules like proteins and nucleic acids, enzymes and coenzymes. These nutrients are therefore limiting in petroleum polluted environments (Leahy and Colwell, 1990). The effect of carbon source concentration on salicylic acid degradation was done by taking the carbon source range of 0-2 % w/v. The optimum carbon source was not obtained.

Effect of nitrogen source

Nitrogen such as peptone, ammonium sulphate, urea, ammonium chloride, yeast extract and beef extract were used. Each flask containing 100 ml of medium were inoculated with 500 ppm salicylic acid, 1% inoculum and incubated at 30°C for 48 hrs. At interval of 24 hrs, 5 ml of sample were withdrawn from each flask and centrifuged at rpm 5,000 for 20 min. The supernatant was used to analyze for degradation activity. The best degradation rate obtained by nitrogen source was optimized. The salicylic acid degradation was achieved when Yeast extract and beef extract were used as the nitrogen source. While other nitrogen source does not play significant role in biodegradation of salicylic acid.

Effect of different concentrations of salicylic acid on degradation

To check the efficiency of salicylic acid degradation by the bacterial isolate was carried out at different concentration of salicylic acid, 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, and 600 ppm. The optimum concentration was 500 ppm optimize.

CONCLUSION

The isolate D1 was efficient in salicylic acid degradation than the other isolates which was isolated from Damanganga effluent. Isolate D1 was able to degrade salicylic acid within 48 hrs of incubation at 30°C under submerged conditions. The isolate D1 was capable to degrade 500 ppm salicylic acid at pH-7 within 48 hrs of degradation respectively. The temperature range of 25°C and 37°C showed better salicylic acid degradation which were optimized. The degradation in medium with yeast extract seem to be more appropriate nitrogen source for the degradation of salicylic acid degradation. The concentration of yeast extract was studied. The optimum concentration obtained was 0.1% yeast extract in production medium and it was optimized. The results show that in the range of 500-1000 ppm the salicylic acid degradation observed was in the range of 92 – 80 % degradation. The effect of incubation period was determined by incubating production medium at 48 hrs, the time needed to achieve the maximum degradation of salicylic acid.

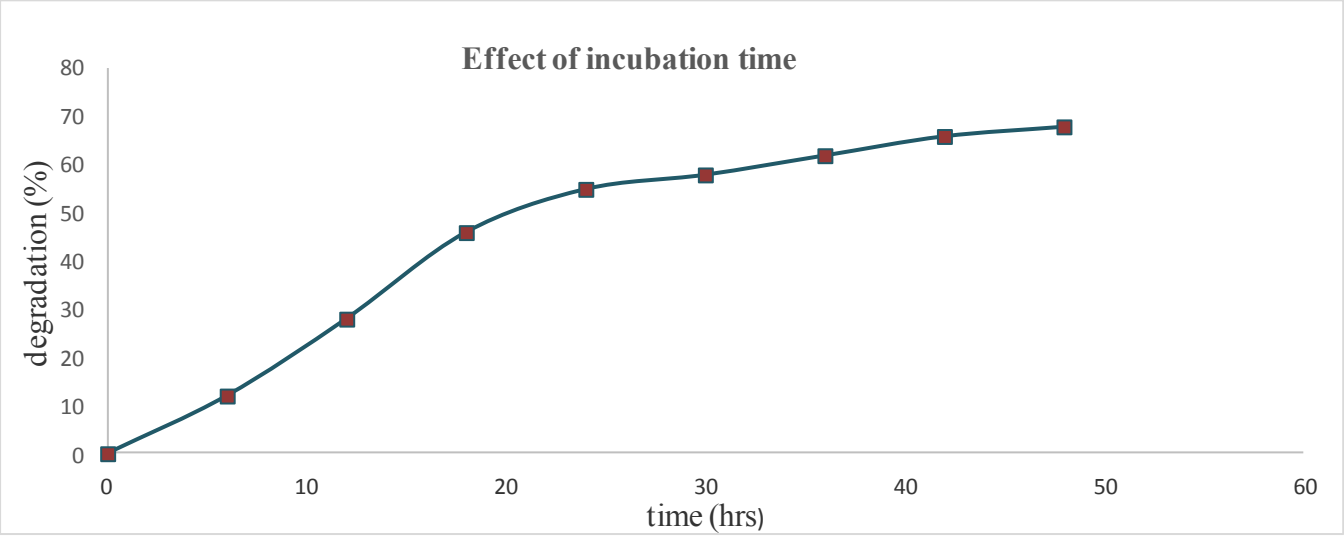

Figure 1: Effect of different incubation period on salicylic acid degradation

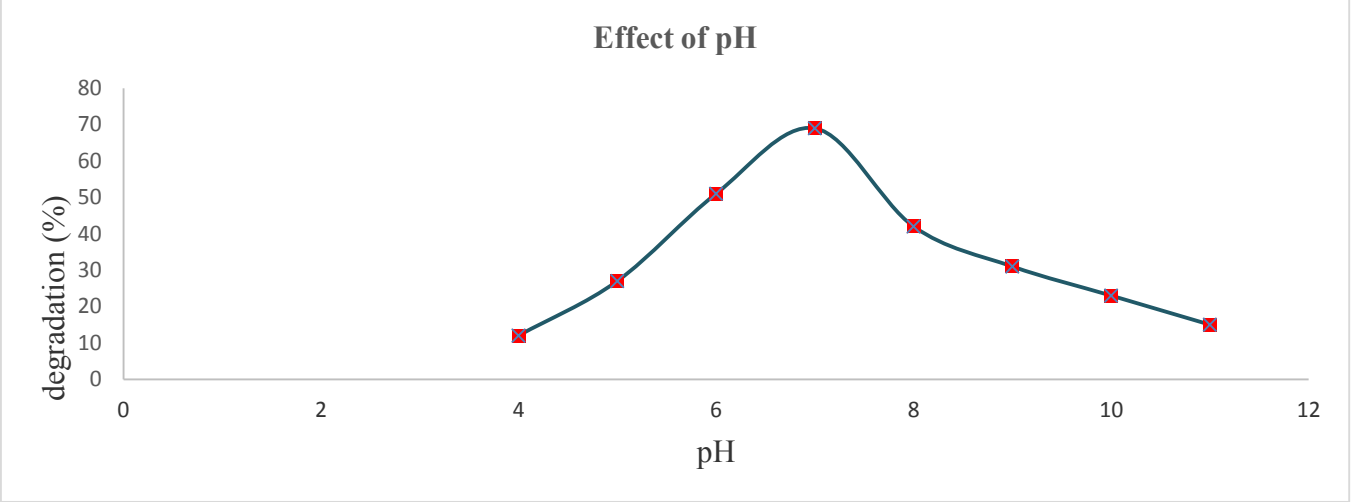

Figure 2: Effect of different pH on salicylic acid degradation

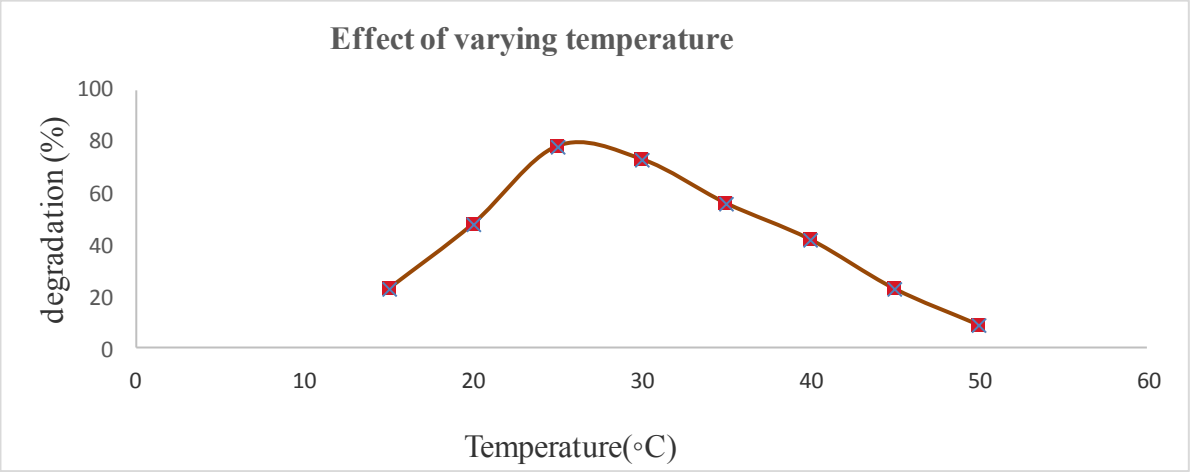

Figure 3: Effect of different Temperature on salicylic acid degradation

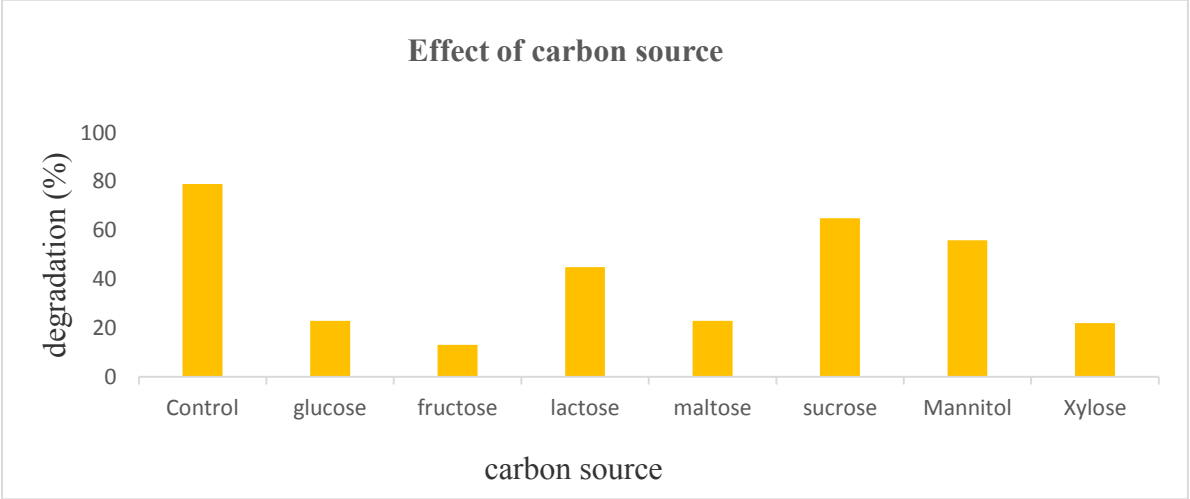

Figure 4: Effect of different Carbon sources on salicylic acid degradation

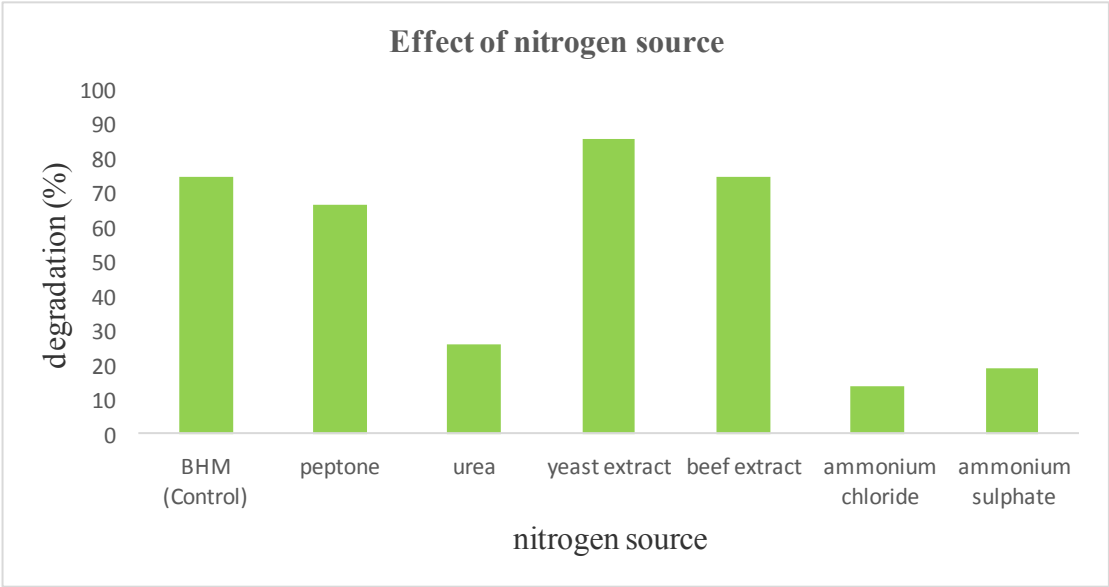

Figure 5: Effect of different nitrogen sources on salicylic acid degradation

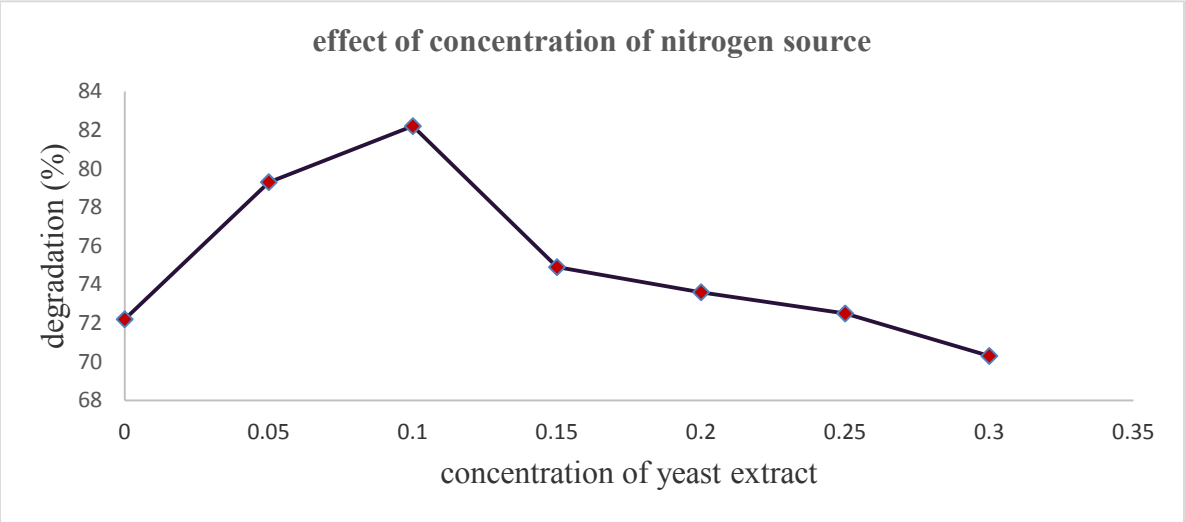

Figure 6: Effect of different nitrogen concentration on salicylic acid degradation

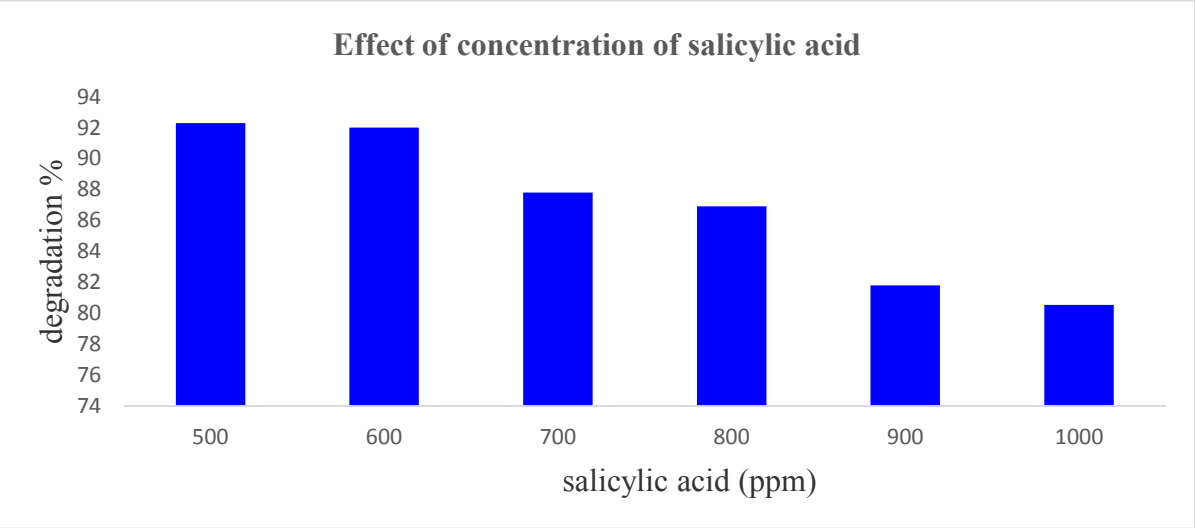

Figure 7: Effect of different concentration of salicylic acid on degradation

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