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RESEARCH ARTICLE

BIOREMOVAL OF INKJET INK AND BIODEINKING OF INKJET INK PRINTED PAPER USING
PSEUDOMONAS SP. OWS1

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ABSTRACT

The utilization of microbes has been suggested as an eco-friendly alternative to traditional chemical deinking in the recycling of used paper. The aim of this study was to decolourize inkjet ink and to achieve deinking of printed paper pulp using *Pseudomonas sp. OWS1* isolated from extreme habitat. Initially the bacterial strains screened for inkjet ink decolourizing and deinking of printed paper pulp were isolated from oil-contaminated effluent water sample and were biochemically characterized. Decolourization of inkjet ink was optimized using various parameters such as pH, glucose concentration, and medium supplemented with 1% starch and 1% Tween 80, inkjet ink concentration, anaerobic and aerobic conditions and cell immobilization. Partial decolourization of the paper pulp was achieved within 5 days at 28 °C. The immobilization of bacterial cells was effective in decolourization of inkjet solution within 2 days at 28 °C. The isolate *Pseudomonas sp. OWS1* has the ability to decolourize the well-known commercial dyes. Preliminary screening for extracellular enzyme was also done. The present investigation clearly demonstrated that decolourization of inkjet ink and deinking of printed paper pulp was more effective by using the culture *Pseudomonas sp. OWS1*. It also has a high potential to decolourize the well-known commercially available dyes.

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INTRODUCTION

There is an increasing demand for paper manufacturing and thus the requirement for plant raw material has also proportionately increased. Increased environmental concerns are creating pressure for adoption of new, eco-friendly technologies (Dhiman *et al.*, 2009). Indeed, recycling of paper is a challenging approach to produce new useful paper products from old or used paper and would definitely reduce such environmental impacts. Recycling of paper requires the removal of the printing ink (deinking) to obtain brighter paper pulp. In conventional method, deinking can be achieved by the chemical bleaching method, but the release of these chemicals after the process often leads to environmental pollution. Several attempts have been made to develop environment friendly approaches for deinking waste paper (Pala *et al.*, 2004). Biological treatment is often the most eco-friendly, economical and alternative when compared with other conventional processes which include both physical and chemical methods. Removal of printing inks and dyes by microorganisms such as bacteria, yeasts, algae and fungi have been reported by several researchers (McMullan *et al.*, 2001; Fu and Viraraghavan, 2001). However, the use of biodegradation as a deinking application is often restricted because of technical constraints. According to Bhattacharyya

and Sharma (2003), biological treatment requires a large land area and is constrained by sensitivity toward diurnal variation as well as toxicity of some chemicals and less flexibility in design and operation. Furthermore, biological treatment is incapable of obtaining satisfactory colour elimination. Moreover, although many organic molecules are degraded, many others are recalcitrant due to their complex chemical structure and synthetic organic origin (Fu and Viraraghavan, 2001; Qurratulane Bari and Nagendra Bhardwaj, 2014). Another deinking environment friendly deinking strategy involves deinking of old newsprint and office paper by using microbial enzymes like xylanases, cellulases, glucanases, lipases, laccases, and mannanase. However, the use of purified enzymes for deinking is slightly expensive.

One of the major issues in deinking is that the both chemical and biological deinking methods do not work efficiently when it comes to inkjet printed papers as inkjet ink is not easily removed. In fact, even with enzymatic deinking, the degree of paper deinking is very less in the case of inkjet printed paper. Decolourization of inkjet ink and deinking of inkjet ink printed paper by using a laccase mediator system composed of commercial *Myceliophthora thermophila* and *Trametes versicolor* laccase and acetosyringone as a mediator compound has been reported by Katariina Nyman and Terhi Hakala (2011). There are only a few attempts made at biological deinking of inkjet printed paper using microorganisms. Deinking and decolourization of the dislodged ink particles

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from inkjet printed paper pulp by a marine bacterium, *Vibrio alginolyticus* isolate no. NIO/DI/32, obtained from marine sediments has been reported by Mohandass and Chandralatha (2005). In this context, the aim of the present study was to develop a better technique for decolourization of inkjet ink and deinking of inkjet ink printed paper which in turn can facilitate a low-cost, environment friendly and publically acceptable biological treatment technology for paper recycling. A bacterial species named as *Pseudomonas* sp. OWS1 had been isolated and used in this study to decolourize inkjet ink and inkjet ink printed paper pulp. *Pseudomonas* sp. OWS1 has been proved to be a potential candidate for successful biological deinking of inkjet ink printed paper.

MATERIALS AND METHODS

Chemicals and Media

Inkjet ink (Refill inkjet kit, Florida), and the commercial dyes (Congo red, Methylene Blue, Orange G) were purchased from Artek Scientific suppliers, Chennai. All microbiological media and media ingredients were purchased from HiMedia Laboratories (Mumbai, MH, India).

Isolation, Screening and identification of inkjet ink degrading bacteria

The inkjet ink decolourizing bacteria were isolated by growth in 0.01% of inkjet ink (Florida inkjet ink) in 100 mL of nutrient broth. The water sample was serially diluted under strict aseptic condition in a laminar flow. 100 μ L of 10^{-6} dilution sample was inoculated in an Erlenmeyer flask, which served as the test flask. For control, 100 μ L of sterile water was inoculated and kept for incubation. The inoculated medium was incubated at 28 °C on a shaker. After 2 days decolourization was visually observed in the test flask. From the test flask 100 μ L of culture was taken and inoculated in nutrient agar medium containing 0.01% inkjet ink and it kept for incubation for 2 days. After 2 days, the decolourization was visually observed on the plate, which was streaked and it was subcultured for every two weeks. Purified isolates were characterized by biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test and Urease test. Gram staining was performed as a Morphological test according to Bergeys Manual of Systematic Bacteriology.

Inkjet ink decolourization optimization

Decolourization of inkjet ink by *Pseudomonas* sp. OWS1 was optimized with different parameters such as different concentrations of inkjet ink, pH, glucose, medium supplemented with 1% starch, 1% Tween 80, 1.5% glucose, anaerobic and aerobic conditions, static and shaking conditions and cell immobilization. Initial experiments were carried out in a flask with loopful inoculum of isolate *Pseudomonas* sp. OWS1 in nutrient broth and the flask without culture served as the control.

All the flasks were incubated at 28 °C under without shaking conditions for 4 days. After 4 days, decolourization of sample was measured.

Varying concentrations of inkjet ink

0.01 to 0.04 % of inkjet ink was mixed with 100 mL of nutrient broth in separate flasks respectively. A loopful culture of isolate *Pseudomonas* sp. OWS1 was inoculated into each flask. These flasks were incubated at 28 °C without shaking.

Varying concentrations of glucose

0.0%, 0.5%, 1.0%, 1.5%, 2%, 2.5% and 3% of glucose were mixed with 100 mL of nutrient broth containing 0.01% inkjet ink in separate flasks respectively. A loopful culture of isolate *Pseudomonas* sp. OWS1 was inoculated into each flask. These flasks were incubated at 28 °C without shaking.

Medium supplemented with 1% starch, 1% Tween 80 and 1.5% glucose

1% starch, 1% Tween 80 and 1.5% glucose was supplemented in 100 mL nutrient broth containing 0.01% inkjet ink in the control and test flask. A loopful culture of isolate *Pseudomonas* sp. OWS1 was inoculated in the test flask. Both flasks were kept for incubation at 28 °C without shaking.

Varying pH

Deinking trials were carried out at pH 4, 5, 6, 7 and 8. 0.01% of inkjet ink was mixed with 100 mL of nutrient broth in separate flasks respectively. A loopful culture of isolate *Pseudomonas* sp. OWS1 was inoculated into each flask. These flasks were incubated at 28 °C without shaking.

Anaerobic and aerobic conditions

0.01% ink, 1% starch, 1% Tween 80, 1.5% glucose in nutrient broth was prepared. It was Sterilized under 15 lbs for 15 min. The flasks were inoculated with a loopful culture of isolate *Pseudomonas* sp. OWS1 and kept for incubation. For anaerobic culture, the flasks were kept in an anaerobic chamber. These flasks were incubated for 4 days at 28 °C without shaking.

Static and shaking conditions

0.01% ink, 1% starch, 1% Tween 80, 1.5% glucose in nutrient broth was prepared. It was sterilized under 15 lbs for 15 min. The flasks were inoculated with a loopful culture of isolate *Pseudomonas* sp. OWS1 and kept for incubation. For static condition it was kept on a static platform and for shaking condition it was kept on a orbital shaker at 100 rpm.

Deinking trials using immobilized bacterial cells

Cells of the *Pseudomonas* sp. OWS1 isolate were immobilized in calcium alginate beads. 1 g of cell pellet was thoroughly mixed with 50 mL of 2% sterile sodium alginate solution. This solution was added drop wise using a sterile pipette into 0.02% of calcium chloride solution aseptically. Calcium alginate beads (without cell pellet) were used as the control. 2 g of the beads was added into 0.01% ink in 100 mL of nutrient broth supplemented with 1% starch, 1% Tween 80, 1.5% glucose and 0.01% inkjet ink. For control, only the calcium alginate beads were incubated with 0.01% inkjet ink in a 100 mL of nutrient

broth. These flasks were kept for incubation at 28 °C without shaking for 2 days.

Deinking trials using inkjet ink containing paper pulp

Inkjet ink coloured paper was prepared by manually applying Florida inkjet ink on the both sides of a normal A4 printing paper and it was dried for 2-3 days. The pieces of paper were cut soaked in hot water for 3 h and macerated in a domestic mixer by adding 0.1% Tween 80, a non-ionic surfactant. The macerated pulp was oven-dried at 40 °C overnight and stored in a sterile container. 3 g of inkjet paper pulp was weighed and added to 100 mL of nutrient broth supplemented with 1% starch, 1% tween 80, 1.5% glucose in separate flasks. The flasks were sterilized by autoclaving. After cooling to room temperature, the flasks were inoculated with a loopful culture of *Pseudomonas* sp. OWS1. The flasks were incubated under aerobic conditions. The incubation was done at 28 °C without shaking. In the control no bacterial inoculum was added. After 5 days the decolourized pulp was visually observed. The decolourized pulp was collected, washed with tap water and filtered using cotton wool. Then the pulp was again macerated with a mortar and pestle and pressed out by hand and dried. The thickness of paper was measured and compared with the control paper.

Decolourization of commercial dyes with well-known structure

0.01% of the different types of commercial dyes (well-known structure) like Orange G, Methylene blue, Congo red were mixed with 1% starch, 1% Tween 80 and 1.5% glucose in nutrient broth in separate flasks. The flasks were sterilized with 15 lbs for 15 min. The flasks were inoculated with Loopful inoculum of *Pseudomonas* sp. OWS1 and kept for incubation without shaking conditions. Every 2 days decolorization was observed in the flasks and measured on spectrophotometrically (Hitachi U-1800) at 452, 663,497 nm. The percentage of decolourization was calculated as follows:

$$\% \text{ Decolourization} = \frac{\text{Initial absorbance (A}_0\text{)} - \text{Absorbance after incubation (A}_1\text{)}}{\text{Initial absorbance (A}_0\text{)}} \times 100$$

Preliminary screening for extracellular enzymes

The active culture of *Pseudomonas* sp. OWS1 was used for the screening of extracellular enzyme activity amylase activity was screened by using starch agar, protease activity was screened by using skim milk agar, lipase activity was screened by using tween 80, chitinase activity was screened by using colloidal chitin agar, gelatinase activity was screened by using gelatin agar and laccase activity was screened by using 0.02% guaiacol containing nutrient agar medium. A loopful culture of isolate *Pseudomonas* sp. OWS1 was streaked on to the agar and it was kept for incubation. The zone of clearance was measured.

RESULTS

Isolation, Screening and identification of inkjet ink degrading bacteria

After 2 days of incubation the decolourization was visually observed on the plate, where the culture was streaked on

nutrient agar medium. The bacterial cultures were identified by microscopy and biochemical tests as shown in Table 2. Based on morphological and biochemical characterization the culture was identified as *Pseudomonas* sp. and was named as *Pseudomonas* sp. OWS1.

Removal of inkjet ink

Influence of concentrations of inkjet ink

Decolourization was observed in flasks containing 0.01% to 0.04% inkjet ink. The maximum decolourization was recorded in the flask with 0.01% inkjet ink on the 2nd day of incubation whereas in flasks containing 0.02 to 0.04% inkjet ink minimal decolourization was observed.

Influence of concentrations of glucose

The isolate OWS1 showed maximum decolourization when the medium was supplemented with 1.5% glucose and this was closely followed by 1% of glucose. The decolourization of inkjet ink increased with increase in the concentration of glucose but declined when the concentration was increased after 1.5%.

Influence of medium supplemented with 1% starch, 1% Tween 80 and 1.5% glucose

The isolate *Pseudomonas* sp. OWS1 showed maximum decolourization when grown in a medium supplemented with 1% starch, 1% tween 80, and 1.5% glucose.

Influence of different pH

The isolate *Pseudomonas* sp. OWS1 showed maximum decolourization at pH 5. At other pH ranges used, the isolate did not show significant decolourization.

Influence of anaerobic and aerobic conditions

It was clearly observed that the bacterial isolate *Pseudomonas* sp. OWS1 has the ability to decolourize the inkjet ink in both aerobic and anaerobic conditions. But compared to anaerobic condition, aerobic conditions showed better decolourization.

Influence of static and shaking conditions

The decolourization was better when the cultures were kept in static conditions as compared to shaking. In shaking condition minimum decolourization was observed and the biomass was also less when compared to static condition.

Deinking trials using immobilized bacterial cells

Deinking of inkjet ink solution by immobilized bacterial cells was effectively enhanced decolourization within 2 days than the non-immobilized bacterial cells. There was no colour change observed in the control flask.

Deinking trials using inkjet ink containing paper pulp

The inkjet ink containing paper pulp inoculated with bacterial culture OWS1 was decolourized on the 5th day of incubation in

aerobic conditions. The results for aerobic deinking are shown in Figures (1 and 2).



Figure 1. Shows deinking of inkjet ink containing paper pulp on 5th day of aerobic incubation

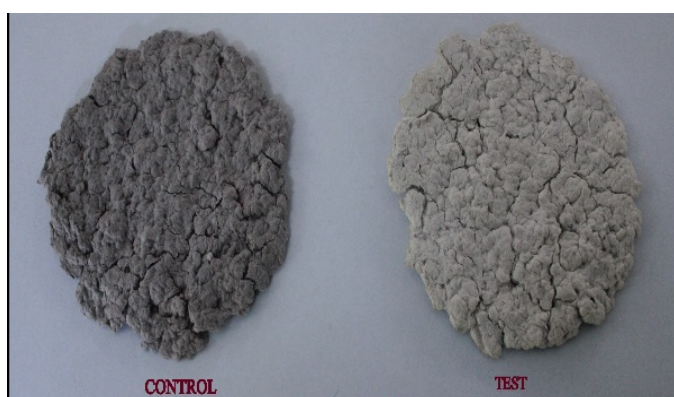
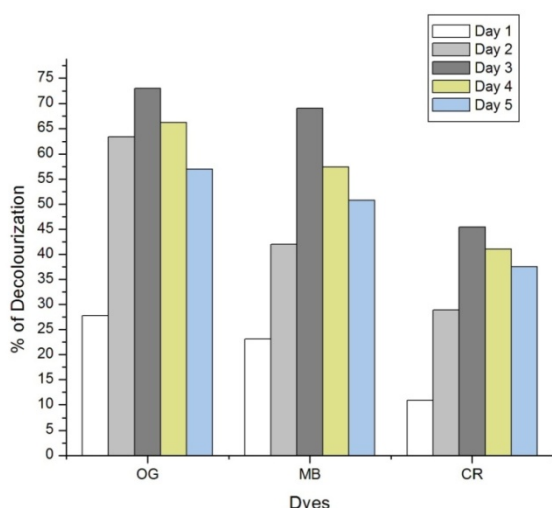


Figure 2. Shows handmade paper of untreated control and biodeinked inkjet printed paper (test)



(OG – Orange G, MB – Methylene Blue, CR – Congo Red)

Figure 3. Shows % of decolourization of commercial dyes

Deinking trials using commercial dyes with well-known structure

The commercial dyes Orange G, Methylene blue, Congo red were decolourized by day 2 without shaking (Figure 3). No decolourization was observed in shake flasks.

Preliminary screening for extracellular enzymes

The isolate *Pseudomonas* sp. OWS1 showed positive activity for all the extra cellular enzymes especially amylase, protease, lipase and laccase shows the maximum zone of clearance as shown in the Table 2.

Table 1. Shows biochemical tests of the culture isolate OWS1

S.No	Biochemical Tests	Results
1.	Gram staining	G-ve
2.	Methyl Red	-ve
3.	Voges Proskauer	-ve
4.	Citrate Utilization	+ve
5.	Carbohydrate Fermentation	+ve
6.	Urease	+ve
7.	Catalase	+ve
8.	Indole	-ve
9.	High Salt Tolerance	+ve

Table 2. Shows Preliminary screening for extracellular enzyme activity

S.No	Enzyme Activity	Results
1.	Amylase	+++
2.	Protease	+++
3.	Lipase	+++
4.	Chitinase	++
5.	Gelatinase	++
6.	Laccase	+++

[+: Positive, (+: 0.1 – 0.5c.m, +++: 0.6 – 1.0 c.m)]

DISCUSSION

The isolate OWS1 has been identified as *Pseudomonas* sp. as per the Bergey's Manual of Systematic Bacteriology. Laccase production may be a contributing factor for the efficiency of OWS1 in decolourizing inkjet ink. Laccase has been indicated in several dye degradation studies. The use of laccase /laccase mediator could be useful in delignification, bioleaching and colour removal (Katariina Nyman and TerhiHakala, 2011; Valls *et al.* 2009). The capability of laccase to oxidize surface lignin from mechanical paper pulp fibers has been reported (Robinson *et al.*, 2001). It has been shown that old newspaper could be deinked with laccase - violuric acid (LVS) and that Laccase treatment resulted in the degradation of most of the extractives (Xu *et al.*, 2009). Decolourization of various concentrations of inkjet ink in a matter of a few days shows that the isolate is able to utilize and metabolize the inkjet ink efficiently as a nutrient source. Several authors have reported better decolourization of dyes in the presence of glucose as a carbon source (Joshi *et al.*, 2013; Oranusi and Ogugbue, 2005). Likewise, in our study also, the isolate *Pseudomonas* sp. OWS1 showed maximum decolourization when the medium was supplemented with 1.5% glucose. The glucose concentration used here is also an important factor for decolourization because at higher glucose concentrations, the bacteria will tend to preferentially use the glucose as a nutrient rather than using the inkjet ink as a result of which there may be less decolourization of the inkjet ink.

The isolate *Pseudomonas* sp. OWS1 showed maximum decolourization when grown in a medium supplemented with 1% starch, 1% Tween 80, and 1.5% glucose as compared to medium which was supplemented with only 1.5% glucose. This may be due to the fact that starch acts as an additional

energy source for the isolate and may therefore lead to an increase in the number of bacterial cells available for decolourization. Tween 80 is a source of unsaturated fatty acids which improves the growth rates and metabolic activity in microbial cells. Tran *et al* (2010) have also shown that fermentation by yeast cells is increased by supplementation of the medium with Tween 80 which increases the number of viable yeast cells in culture. This may hold good for the isolate *Pseudomonas* sp. OWS1. The bacterial isolate *Pseudomonas* sp. OWS1 is unique in that the maximum decolourization of inkjet ink was observed at an acidic pH 5. This also indicates that the enzymes involved in the decolourization of inkjet ink are working maximally at this acidic pH. This may be advantageous in industrial settings during paper recycling. Many authors have also reported that decolourization of dyes by bacteria is better in acidic pH conditions (Tripathi and Srivastava, 2011; Kuo-cheng chen *et al.*, 2003). The ability of *Pseudomonas* sp. OWS1 to decolourize the inkjet ink in both aerobic and anaerobic conditions, suggests that the levels of oxygen available during the growth of the isolate has no effect on the metabolic activity of the isolate. The decolourization was also better when the cultures were kept in static conditions which may indicate that the enzymes required for decolourization are produced more in static conditions under low oxygen conditions or probably that high oxygen levels may be competing with the inkjet ink degradation products for electron carriers under aerobic conditions. There are several reports that decolourization of dyes by bacteria such as *Pseudomonas* spp. is better in static conditions (Shah *et al.*, 2013).

The increase in the rate of decolourization after immobilization of *Pseudomonas* sp. OWS1 may be due to the higher cell concentrations and greater enzyme stability when cells are immobilized. There are several reports that confirm that the efficiency of dye degradation is higher when immobilized cells rather than when free cells are used. According to Mohandass and Raghukumar (2005), bacterial cells immobilized in calcium alginate beads were able to decolourize inkjet-printed paper pulp within 72 h. The cell-free culture supernatant of the bacterium grown in nutrient broth was not effective in deinking. Use of immobilized cells for deinking of paper pulp is also a more hassle-free approach as cells can be easily separated from the pulp during further recycling processes and even be reused. The isolate *Pseudomonas* sp. OWS1 is also efficient in deinking the inkjet printed paper pulp simultaneously enhancing the visual quality of the paper and this is due to the many enzymes it may be actively secreting during growth. The isolate *Pseudomonas* sp. OWS1 also shows excellent potential for degradation of the commercial dyes methylene blue, orange G and Congo red which may structurally resemble dyes used in paper printing. The types of chemical structures and functional groups that the isolate is able to act upon during degradation can be studied by further screening for degradation of more commercial dyes using this isolate. That *Pseudomonas* spp also has the ability to decolourize the dyes was reported by Ponraj *et al* (2011) and Sneha *et al* (2013). The isolate is positive for some extracellular enzymes such as amylase, lipase, chitinase, gelatinase, laccase, protease for which it was screened, as these enzymes are expected to be involved in the degradation of dyes and recycling of printed paper based on previous literature.

Many authors have reported deinking of office waste paper using hemicellulase and endoglucanase (Gubitz *et al.*, 1998; Marques *et al.*, 2003), amylase (Bajpai *et al.*, 1999), cellulase (Neal *et al.*, 1994). Morkbak *et al.*, (1999) also reported removal of oil-carrier-based inks can be facilitated by treatment with lipases and esterases. Deinking and decolorization using a mixture of commercially available amylase and laccase has been reported by Frank (2001). This preliminary study therefore confirms that the isolate *Pseudomonas* sp. OWS1 is a potential applicant for use in biological deinking of inkjet ink from printed paper.

Conclusion

The current study clearly concludes that *Pseudomonas* sp. OWS1 has a significant role to play in the decolourization of inkjet ink solution and deinking of inkjet ink paper pulp. This bacterial isolate also shows good decolourizing capacity of commercial dyes. The bacterial isolate OWS1 also showed better decolourizing capacity when it was immobilized. The active culture of the OWS1 has an efficient enzyme activity. The results obtained by this study indicate that the isolate OWS1 will be a promising candidate for utilize in the paper industry as well as in bioremediation of dyes.

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