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ORIGINAL PAPER

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Biological deinking of inkjet-printed paper using *Vibrio alginolyticus* and its enzymes

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Abstract Recycling of office waste paper (photocopy, inkjet, and laser prints) is a major problem due to difficulty in removal of nonimpact ink. Biological deinking of office waste paper is reported using several microorganisms and their enzymes. We report here deinking and decolorization of the dislodged ink particles from inkjet printed paper pulp by a marine bacterium, *Vibrio alginolyticus* isolate no. NIO/DI/32, obtained from marine sediments. Decolorization of this pulp was achieved within 72 h by growing the bacterium in the pulp of 3–6% consistency suspended in seawater. Immobilized bacterial cells in sodium alginate beads were also able to decolorize this pulp within 72 h. The cell-free culture supernatant of the bacterium grown in nutrient broth was not effective in deinking. However, when the culture was grown in nutrient broth supplemented with starch or Tween 80, the cell-free culture supernatant could effectively deink and decolorize inkjet-printed paper pulp within 72 h at 30°C. The culture supernatant of *V. alginolyticus* grown in the presence of starch or Tween 80 showed 49 U ml^{-1} and 33 U ml^{-1} amylase and lipase activities, respectively. Dialysis of these culture supernatants through 10 kDa cut-off membrane resulted in a 35–40% reduction in their efficiency in decolorizing the pulp. It appears that amylase and lipase effectively help in dislodging the ink particles from the inkjet printed-paper pulp. We hypothesize that the bacterium might be inducing the formation of low molecular weight free radicals in the culture medium, which might be responsible for decolorization of the pulp.

Keywords Nonimpact ink · Inkjet prints · Marine bacterium · Deinking · Decolorization · Free radicals

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Introduction

The continuously growing paper manufacturing industry imposes a severe demand on green plants that forms the basic raw material. Thus, it is not an environment-friendly option. Recycling of used paper is an alternative that can alleviate this stress exercised on the environment. The three major sources of raw material for such recycling are newsprint, photocopied paper, and inkjet-printed papers. Recycling of paper requires the removal of the printing ink, also called deinking, from the used paper to obtain brighter pulp. Printing on paper is generally carried out by using impact or nonimpact ink. Impact ink, generally used for newsprint does not fuse with the paper and therefore is easy to remove or disperse during the deinking process [13]. This process is now well known and has been carried out in most of the paper mills where newsprint is recycled. On the contrary, nonimpact inks used in photocopying, inkjet, and laser printing results in the ink fusing with the paper and making it non-dispersible, thus rendering the deinking process much more difficult [6]. The toners used for printing generally contain carbon black as the colorant, resin (as a binder), water (as solvent), surfactants (to reduce surface tension), humifactant, buffering agent, and fungicides (http://www.denison.edu/chem/DCS/journal/pellettv1n1.html).

Various hydrolytic enzymes such as cellulases, glucanases, amylase, lipase, and xylanase of bacterial or fungal origin [9, 10, 16, 19] and more recently oxidative enzyme laccase [2] have been used, individually or in combination for deinking of office waste paper. In most of these cases, the fibers with ink liberated from the cellulose matrix float on the surface and these are removed using different floatation devices. However, the pulp needs to be washed thoroughly to remove the detached ink particles. We present results of a process wherein by using the marine bacterium *Vibrio alginolyticus*, isolate no. NIO/DI/32 and its cell-free culture

supernatant, the ink particles are dislodged and simultaneously decolorized.

Materials and methods

Isolation and culture conditions of bacteria

Bacteria were isolated from marine sediments collected from a depth of 2 m in Zuari estuary in Goa (15°30′ N; 73°50′ E) by using selective enrichment technique. In this method, pieces of inkjet-printed paper were buried in 10–15 g of marine sediments and topped with 2.5 l of seawater collected from the same site, thus creating a microcosm environment for the experiment. Bacteria were isolated from these paper pieces at an interval of 1 week up to a period of 5 weeks. Briefly, the procedure included homogenization of a few paper pieces in sterile seawater and an aliquot of 100 µl was spread-plated on nutrient agar medium (HIMEDIA Pvt. Ltd, India) prepared with half-strength seawater. The bacterial cultures thus obtained were maintained on nutrient agar medium prepared with half-strength seawater. The salinity of seawater at the time of collection of sediment was 15 ppt, which is roughly equivalent to half-strength seawater.

Deinking trials using bacterial cells

The bacterial cultures were grown in nutrient broth prepared with half-strength seawater for 3–4 days at 30°C. The bacterial cells were collected by centrifugation under sterile conditions, diluted appropriately to get optical density of 1 at 660 nm using a spectrophotometer (UV/V1201, Shimadzu, Japan).

Inkjet-printed paper from HP printer (printed with single line spacing on both sides) were pulped by soaking in hot water for 2 h, macerated in a domestic mixer after adding 0.1% Tween 80, a non-ionic surfactant. The macerated pulp was oven-dried at 50°C and stored in sterile container under refrigeration.

Immediately before use the pulp was soaked in water for 30 min. Pulp at a consistency of 3–6% (3–6 g pulp in 100 ml of half-strength seawater) was sterilized by autoclaving. After cooling it to room temperature it was inoculated with 10% bacterial inoculum containing 10^8 cells ml^{-1} and incubated at room temperature. After 3–4 days the decolorized pulp was washed thoroughly with tap water. The washed pulp was filtered over a Buchner funnel under suction to obtain the pulp in the form of hand sheets. The hand sheets thus obtained were pressed flat between two stainless steel plates and oven-dried at 50°C for 12 h. They were gently pressed with steam iron to get uniform thickness. Printed paper pulp without treatment with bacterial culture was used as control. Brightness index (BI) was measured as per TAPPI test method T452/ISO2470 protocol using Premier Colors Scan-Spectroscan 5100, USA (at Pudumjee Pulp and Paper Mills Pvt. Ltd., Pune, India).

Deinking trials using immobilized bacterial cells

One of the bacterial isolates, NIO/DI/32 that showed effective deinking of the pulp was used for further studies. Bacterial cells of this isolate were immobilized in sodium alginate. Briefly, this involved mixing 10 ml of cells (containing 10^8 cells ml^{-1}) with 25 ml of 2% alginic acid (Sigma Chemicals, USA). Using an autopipette of 200 µl capacity, the alginic acid solution was dropped into 0.2 M calcium chloride solution that resulted in the formation of beads which were cured overnight at 5°C. Paper pulp from inkjet printed paper was incubated with immobilized bacterial cells for 1–4 days and BI was measured as described earlier.

Deinking trials using cell-free bacterial culture supernatants

The culture was grown in nutrient broth supplemented with 1% starch or Tween 80 or with both. The 5-day-old cultures were centrifuged and the clear filtrate was used as cell-free culture supernatant. The pulp from inkjet-printed paper prepared as described earlier at 3–9% consistency was incubated with 50 ml of the cell-free culture filtrate for 1–4 days. Hand sheets were made with such treated and untreated pulp as described earlier and the BI was measured.

Estimation of enzyme activity in the culture filtrate

The active principle involved in deinking of pulp using the cell-free culture supernatant was investigated. Accordingly, amylase and lipase activities were estimated in the culture filtrate from cultures grown in starch and Tween 80, respectively. Amylase activity was measured colorimetrically using 1% starch, iodine, and potassium iodide [8]. Lipase activity was measured using the model substrate 4-methylumbelliferyle-butyrate (Sigma Chemicals, USA). The fluorescent MUF product released is measured at excitation and emission wave length of 364 and 445 nm, respectively [5]. Laccase activity was determined qualitatively by incorporating the substrate 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid in glycine-HCl buffer at pH 3.0 in nutrient agar medium [12]. Appearance of green zone around the bacterial colony indicates laccase positive reaction. Cellulase and xylanase in the culture supernatant were estimated by growing the cultures in the presence of 1% CMC-cellulose and birchwood xylan (Sigma Chemicals). Enzyme activity was measured by estimating the reducing sugars released using dinitrosalicylic acid reagent as described earlier [14].

Determination of physical and mechanical properties of deinked paper

The drainage rate (ISO 5267/1), burst (ISO 2758) and tear indices (ISO 1974), and tensile strength (ISO 1924/2) of hand sheets made with the pulp after treatment with culture supernatants were measured following the standard procedures.

Results

Identification of bacterial isolate

About 50 bacterial isolates were obtained from microcosm experiment involving incubation of pieces of photocopied and inkjet printed papers in estuarine sediments. Out of the total 50 isolates tested for deinking of inkjet printed paper pulp, an isolate designated NIO/DI/32 alone showed deinking of such pulp within 4–5 days. This isolate was identified as *Vibrio alginolyticus* using *16 s rRNA* gene sequencing data (MIDILABS, Newark, USA) and deposited in the ARS Patent Collection, USDA, Illinois on February, 13, 2003 under the accession number NRRL-B-30638. The culture was maintained on nutrient agar medium prepared with half-strength seawater.

Deinking trials using bacterial cells

The inkjet-printed paper pulp inoculated with bacterial culture NIO/DI/32 was completely decolorized by day 3 (Fig. 1). By increasing the concentrations of bacterial inoculum from 2 ml to 10 ml, the BI of the pulp showed only marginal increase (BI of untreated pulp was 10.6 and that of treated pulp was between 49 and 51 at different concentrations of the bacterial inoculum). By inoculating the pulp with 10% bacterial inoculum, the maximum brightness was achieved by 48 h (Fig. 2). The bacterial culture grown in pulp suspended in tap water, 50% diluted seawater, or 100% seawater was equally effective in deinking and decolorization of the pulp (Fig. 3). Heat-killed cells were not effective in deinking the pulp.

Deinking trials using immobilized bacterial cells

Pulp prepared with inkjet-printed paper at 3% consistency was incubated with sodium alginate beads containing bacterial cells equivalent to 10 ml of inoculum (10^8 cells ml^{-1}) for 72 h at room temperature. The alginate beads without the bacterial cells served as control. The same batch of immobilized cells were used in a second round of deinking trial. The results showed that immobilized cells were as effective as free bacterial cells for deinking purpose (Table 1) and they could be used for two cycles of deinking trials.

Deinking trials using cell-free bacterial culture filtrate

Pulp prepared with inkjet-printed paper when incubated with cell-free culture supernatant of NIO/DI/32 grown in the nutrient broth was not deinked. However, the culture supernatants from the bacterial culture grown in nutrient broth supplemented with starch or Tween 80 were effective in deinking and decolorization of the pulp (Table 2). Pulp used at 6% consistency treated with cell-free culture supernatant of *V. alginolyticus* (NIO/DI/32) grown in the presence of Tween 80 showed maximum increase in BI (Table 2). Deinking of pulp used at 3% consistency treated with culture supernatants was better

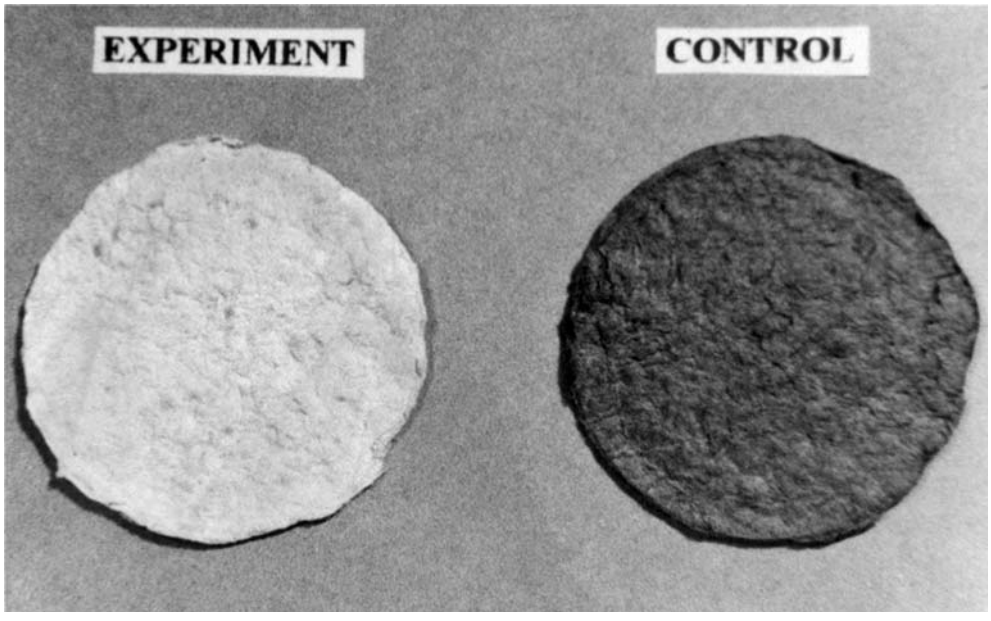

Fig. 1 Hand sheets of biologically deinked inkjet-printed paper and untreated control

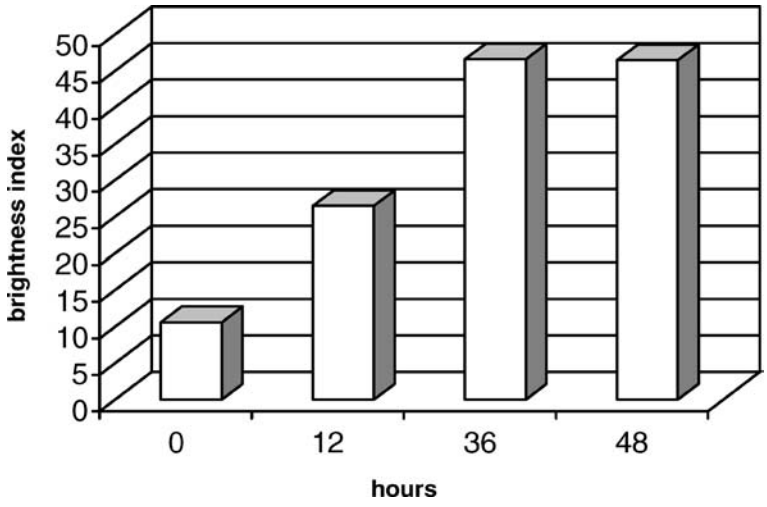

Fig. 2 Brightness index of pulp inoculated with 10% bacterial inoculum after various time intervals

at room temperature than at 37°C (Table 2). Heat-inactivated culture supernatants (either from starch or Tween 80-grown cultures) were not effective in deinking of the pulp. Culture supernatants stored at room temperature for 2 days lost their efficiency for deinking as compared to the fresh culture supernatants. On the other hand freeze-dried culture supernatants resuspended in seawater could effectively deink the pulp (Table 2). A reduction in decolorizing efficiency was noticed when the culture supernatants used were dialyzed using 10 kDa cut-off membrane (Table 2).

Estimation of enzyme activity in the culture supernatants

The culture supernatant from 5-day-old culture of NIO/DI/32 grown in the presence of starch or Tween 80 showed 49 U ml^{-1} amylase activity and 33 U ml^{-1} lipase activity, respectively. These were the minimum concentrations required for complete bleaching of the office waste within 3–4 days at 3–6% pulp consistency. Diluting the enzyme solution did not result in effective deinking.

Laccase and cellulase activity were not detected in the culture supernatant of the bacterial isolate NIO/D1/32.

Physical and mechanical properties of the enzyme-treated and untreated pulp

The tensile, burst and tear indices for untreated non-impact ink printed pulp was 6.84 N m g^{-1}, 0.24 kPa m^2 g^{-1}, and 6.37 mN m^2 g^{-1}, respectively, whereas the values for enzyme-treated pulp were 10.90, 0.45, and 3.46, respectively. The treated pulp thus showed increased tensile and burst indices and a lowered tear index indicating improved physical and mechanical properties of the biologically deinked paper. The

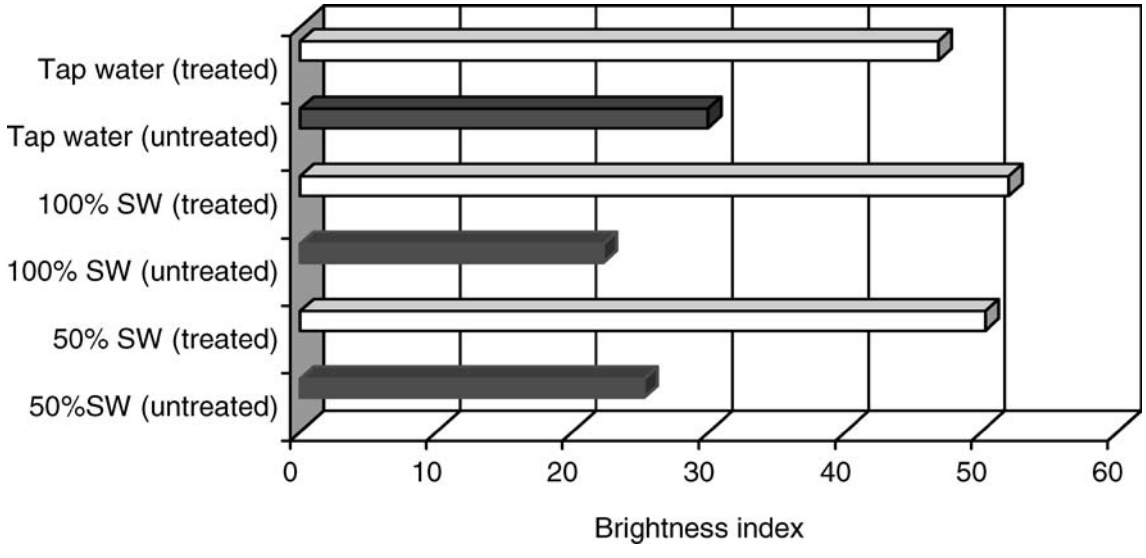

Fig. 3 Brightness index (after 48 h) of pulp suspended in different waters and inoculated with 10% bacterial inoculum

Table 1 Deinking of inkjet-printed paper pulp using various treatments

Treatment	Brightness index
Untreated pulp (control)	20.3
Pulp treated with bacterial cells	50.3
Pulp treated with immobilized bacterial cells (cycle 1)	47.1
Pulp treated with immobilized bacterial cells (cycle 2)	43.0

drainage rates for untreated and treated pulp were 20 and 16 °SR. A decrease in °SR indicates improved drainage of the pulp.

Discussion

The results of the present study clearly indicate that the bacterial cells of *V. alginolyticus*, free or immobilized and cell-free culture supernatants show tremendous potential for environmental friendly technique of biological deinking. Totally decolorized inkjet printed pulp (of 3–6% consistency) is obtained within 48 h of incubation with 10% of bacterial inoculum containing 10^8 cells ml^{-1} (Fig. 1). Treatment with cell-free culture supernatants containing amylase and lipase activity or both resulted in several fold increase in brightness. A major advantage of using *V. alginolyticus* cells or its culture supernatant over other enzyme-based techniques reported is that the ink particles that are dislodged get simultaneously decolorized, thus saving time and water used for floatation and removal of ink particles.

Several authors have reported deinking of office waste paper using hemicellulase and endoglucanase [4, 17] cellulase [11], and amylase [1]. Removal of oil-carrier-based inks can be facilitated by treatment with lipases and esterases [10]. More recently deinking and decolorization using a combination of commercially available amylase and laccase has been reported [2]. The invention described a method for dislodging the ink particles from paper pulp using hemicellulase, lipase, pectin methyl esterase, protease, amylase, and xylanase in the presence of ionic or anionic surfactants. The decolorization of stilbene dye in the ink particles is achieved by using fungal laccase in the presence of chemical mediators like *N*-hydroxybenzothiazole (HBT), violuric acid or 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS). The chemical mediator acts as redox mediator to effectively shuttle electrons between the laccase and the dye [2]. However, the absence of laccase activity in our culture rules out this possibility.

Hemicellulases, cellulases, endogluconases, and xylanases act directly on cellulose fibrils and hydrolyze the fiber-ink bonding regions, which facilitate ink detachment. These enzymes also remove small fibers from the surface of the detached toner/ink particles thus allowing their smooth passage to the top during floatation process. Once the ink particles float on the surface they are removed by a skimming action.

Our culture did not produce cellulase and thus no significant reduction in weight of the pulp was noticed after treatment with bacterial cells. Thus it appears that the starch used as binder in the paper might be hydrolyzed by amylase activity and the olefins present in the ink might be acted upon by lipase activity present in the culture filtrate. Combined action of these two enzymes help in the detachment of ink particles. Decolorization of these particles probably is carried out by free radicals of low molecular weight produced in the culture medium. Therefore, dialysis of these culture supernatants

Table 2 Deinking of inkjet-printed paper pulp using various treatments

Culture supernatant of the bacterium NIO/DI/32 grown in nutrient medium (supplemented with starch or Tween 80 at 1% concentration) for 4 days was added to paper pulp of various consistencies. Amylase and lipase activity in the culture supernatant were 49 U ml^{-1} and 33 U ml^{-1}, respectively. The freeze-dried culture supernatant was diluted to the original volume with 50% diluted seawater and incubated with pulp

Pulp concentration and temperature of incubation	Treatments	Brightness index
3%, 30°C	Untreated pulp (control)	10.6
	Amylase (after dialysis)	50.1 (33)
	Lipase (after dialysis)	66.2 (39.6)
	Amylase + Lipase	56.7
6%, 30°C	Untreated pulp (control)	22.0
	Amylase	47.3
	Lipase	56.1
	Amylase + Lipase	51.7
9%, 30°C	Untreated pulp (Control)	15.9
	Amylase	47.6
	Lipase	55.2
	Amylase + Lipase	51.4
3%, 37°C	Untreated pulp (control)	21.7
	Amylase	57.7
	Lipase	52.6
	Amylase + Lipase	50.2
3%, 30°C	Pulp treated with resuspended freeze-dried culture supernatant (showing amylase activity)	45.3
	Pulp treated with resuspended freeze-dried culture supernatant (showing lipase activity)	42.0

resulted in a loss of these low molecular weight compounds and thus reduced the efficiency of decolorization of the pulp. The role of free radicals in the treatment of various effluents, domestic as well as industrial is well documented [15]. Low molecular weight compounds produced by wood-decaying fungi are known to play an important role in the degradation of wood [3].

The enzymatic treatment contributed to the improvement of the strength properties of the treated pulp compared to the untreated control. Similar improvement in physical and mechanical properties of enzyme-treated pulp has been reported [6, 7]. The decreased drainage rates in enzyme-treated pulp results in faster machine speed resulting in significant savings in energy and cost [1].

Several authors have used highly purified or concentrated enzymes for deinking purpose [7]. However, in our case, the crude unconcentrated culture supernatant alone could bring about deinking and decolorization of office waste paper used at 3–6% pulp consistency within 48 h of incubation. Cell-free culture supernatant of the bacterium grown in nutrient broth alone was not effective in deinking and only when supplemented with starch or Tween 80 was it effective in deinking, which clearly shows that the enzyme amylase and lipase/esterase induced are key players in dislodging of ink particles. Absence of deinking by using heat-killed culture supernatant and effieciency of freeze-dried culture filtrate in this process further strengthens the role played by the enzymes in the deinking process. Subsequent decolorization of the dislodged ink particles is perhaps brought about by low molecular weight compounds that generate free radicals.

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