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Impact of Untreated Brewery Effluent on Bacteriological Characteristic of Agricultural Soil

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ABSTRACT

This research was conducted to evaluate the impact of untreated brewery effluent on the bacteriological characteristic of agricultural soil. This is to ascertain the suitability of untreated brewery effluent for irrigation purposes. Untreated brewery effluent was aseptically collected from Intafact brewery, Onitsha, Anambra State, Nigeria., while agricultural soil was obtained from a maize farm in Onitsha. Five kilograms of the soil sample was treated with 200 ml of the effluent in a pot experiment and allowed for 80 days. The bacteriological characteristic of the effluent and soil samples (treated and untreated) was determined by estimating the total bacterial count, total coliform count, fecal coliform count, *Pseudomonas* count, Rhizobial count and *Clostridium* count. Results obtained showed that the untreated brewery effluent had total bacterial count of 5.2 × 10^3cfu/ml, total coliform count of 0.5× 10^3cfu/ml, *Pseudomonas* count of 0.7 × 10^3cfu/g and zero fecal coliform, *Rhizobium* and *Clostridium* counts. The total bacterial count, total coliform count, *Pseudomonas* count, rhizobial count and *Clostridium* count for the untreated and treated soil samples were 10.8× 10^3cfu/g and 22.8× 10^3cfu/g, 7.2× 10^3cfu/g and 11.4× 10^3cfu/g, 0.9 × 10^3cfu/g and 2.5 × 10^3cfu/g, 0.4× 10^3cfu/g and 0.73× 10^3cfu/g, 1.2 × 10^3cfu/g and 8.0 × 10^3cfu/g respectively. A total of 34 bacterial organisms belonging to ten different genera - *Pseudomonas, Bacillus*, *Azotobacter*, *Streptococcus*, *Staphylococcus*, *Rhizobium*, *Proteus*, *Enterobacter*, *Klebsiella* and *Serratia* were isolated from all the samples analyzed. While *Enterobacter* sp. occurred most in the effluent sample, *Bacillus*sp predominated in the untreated soil sample. *Rhizobium* and *Pseudomonas* count were significantly higher (p-value < 0.05) in the treated soil sample. This study showed that untreated brewery effluent enhanced the growth of plant growth promoting bacteria, hence, can serve efficiently as irrigation water.

KEYWORDS: *brewery, effluent, untreated soil, bacteria, Rhizobium, irrigation*

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1. INTRODUCTION:

Wastewater effluents are major contributors to a variety of water pollution problems. Most cities of developing countries generate on the average 30 – 70 × 10^6litres of wastewater per person per year. Owing to lack of or improper wastewater treatment facilities, wastewater and its effluents are often discharged into surface water sources, which are receptacles for domestic and industrial wastes, resulting to pollution. The poor quality of wastewater effluents is responsible for the degradation of the receiving surface water bodies (Edokpayi*et al*., 2017).

Irrigation with effluent could not only significantly relieve pressure on fresh water resources, but also alleviate their discharge into water environment, avoiding in this way the deterioration of fresh water ecosystems associated with eutrophication and algal bloom (Toze, 2006). The use of wastewater in irrigation has also been found to have additional

agronomic benefits associated with soil structure and fertility. This is especially important in arid and semiarid regions where commonly scarcity of fresh water forces farmers to use water from other sources to cultivate thirsty crops (Fedorof,2010). On the other hand, while it's known that treated wastewater is a good way to replace freshwater resources in agriculture and as a natural fertilizer, few studies have already assessed the impact of these non-conventional water on soil's microbial communities and their activities (Ibekwe*et al*., 2018). The soil is a key natural resource interacting with above-ground plant and animal communities and contributing to a better functioning (Ibekwe*et al.*, 2017).

Irrigation with wastewater is also associated with several biological risks; the presence of pathogens, viruses, helminths and protozoa in soils (Sacks, *et al*. 2011), which have opened a new controversial front in the public debate (Pham, *et al*. 2011). Hidri*et al*. (2014), found that long-term irrigation with treated wastewater resulted in increased soil microbial abundance and induced in particular, compositions of the bacterial and fungal communities. Oved *et al*. (2001) and Ndour*et al*. (2008), investigated that wastewater irrigation produced shifts in ammonia-oxidizing bacteria population in soils, as compared to soils irrigated with freshwater. Therefore, waste effluent may contain bacteria that may be very active in many soil functions as well as some potential pathogens. According to Kizilglu*et al*. (2007), wastewater has a high nutritive value that improves plant growth. It had been shown that soil irrigated with wastewater contained 4.1% of organic particles by weight, but these particles harbored up to 47.8% of the total soil carbon and 41.7% of nitrogen, and thus, represented an important storage of energy and nutrient for microorganisms (Filip, 2000).

Despite the obvious benefits of wastewater irrigation, the human and environmental health recorded many concerns of this process (Phung *et al*., 2011). Many reports correlated the relation between fresh vegetables and foodborne diseases outbreaks that has led to concerns about contamination of vegetables with fecal pathogenic bacteria in the agricultural environment (Tauxe*et al*., 1997). Application of contaminated irrigation water to soil also represents possible sources of contamination. However, standards are required to ensure safe use of wastewater and to avoid biological risks to the human population.

The potential for brewery wastewater use is enormous, but its impact on plant production, soil physical and hydraulic properties, and microbial composition, soil quality, and subsequently public health require considerable investigation. Therefore, this study aims to investigate the impact of untreated brewery effluent on the bacteriological characteristic of agricultural soil.

2. MATERIALS AND METHODS

2.1. Sample Collection

2.1.1. Collection of untreated Brewery effluent

Untreated brewery effluent from Intafact Beverages Ltd. Onitsha, Anambra State Nigeria, was collected using a 1-liter sterile plastic container. The sample container was first rinsed with the effluent several times before collection. The sample was transported to Microbiology Laboratory of the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka for bacteriological analysis.

2.1.2. Soil sample collection

The soil sample for the pot experiment was collected using a sterile soil auger at a depth of 15 cm in a sterile sample bag from an Agricultural farm at Government House Awka.

2.2. Preparation of soil sample

The soil sample was prepared according to the method described by Motsara*et al*. (2008). Five kilograms of the prepared soil was weighed into a plastic container and treated weekly with 200ml of untreated brewery effluent. The control sample was also set up and treated with same volume of tap water on weekly basis as described by Sesan*et al.* (2013). All the samples were allowed to stay for 80 days while exposing them equally to same environmental condition.

2.3. Bacteriological analyses of the untreated brewery effluent and soil samples before and after treatment

The bacteriological analyses of the brewery effluent and soil samples were performed in the Microbiology Laboratory of the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria following the procedures described by Cheesbrough (2006). Total bacterial counts (TBC), total coliform count (TCC), fecal count (FC), total *Pseudomonas* count (TSC), total *Clostridium* count and total *Rhizobuim* count (TRC) were determined using the methods of Cheesbrough (2006).

2.4. Culture media preparation

The culture media used were Nutrient agar, MacConkey agar, Eosin Methylene Blue (EMB), Cetrimide agar, Yeast Extract Mannitol agar and Reinforced Differential Clostridial medium. They were prepared according to the manufacturers' specifications.

2.5. Total bacterial count

The effluent samples were homogenized by shaking them for twenty -five times. The soil samples were also sieved and homogenized. The samples were serially diluted by ten-fold and 1ml of the 10^{-2} dilution factor (of effluent and soil) was inoculated onto nutrient agar plate by pour plating technique. The plates were incubated at 37^0C for 24 hours and colonies that developed on the plates were counted and recorded as colony forming unit. The bacterial colonies were further sub-cultured in freshly-prepared nutrient agar plates to obtain pure cultures which were stored on nutrient agar slants for characterization and identification.

2.6. Total coliform count

The samples (soil and effluent) were serially diluted and 1 ml of 10^{-2} dilution factor was inoculated by pour plate technique onto MacConkey agar plates. The inoculated plates were incubated at 37^oC for 48 hours. Pink colonies that developed after 48 hours were counted and recorded as coliform colony forming unit. Each colony was sub-cultured and stored on a sterile nutrient agar slant for characterization and identification.

2.7. Fecal coliform count

The samples (soil and effluent) were serially diluted and 1 ml of 10^{-2} dilution factor was inoculated by pour plate technique onto Eosin Methylene Blue (EMB) agar plates. The inoculated plates were incubated at 44.5^oC for 48 hours. Green metallic sheen colonies that developed after incubation were counted and recorded as fecal coliform colony forming unit.

2.8. Pseudomonas count

Pour plating technique was used to determine the Pseudomonas count of the samples $.10^{-2}$ dilution factor from the brewery effluent sample soil samples after ten-fold serial dilutions were separately introduced into properly labelled sterile Petri-plates. Sterilized cetrimide agar at 45^0C was dispensed into the plates, gently swirled and incubated at 37^oC for 24 hours. The lemon to green discrete colonies of *Pseudomonas* species were counted and recorded.

2.9. Clostridium count

One ml of 10^{-2} dilution factor of the diluted effluent sample and soil samples were inoculated onto sterile Reinforced Differential Clostridial agar plate supplemented with nystatin, and incubated an aerobically using an anaerobic jar at 25^oC for 72 hours. The black colonies that developed were counted and recorded.

2.10. *Rhizobium* count

One ml of 10^{-2} dilution factor of the diluted effluent sample and soil samples were inoculated into sterile Yeast Extract Mannitol Agar (YEMA) plate, and incubated at 37^oC for 7days. The large mucoid elevated colonies of *Rhizobium species* that developed were counted, sub-cultured and stored on sterile nutrient agar slants for characterization and identification.

2.11. Characterization and Identification of the Bacterial Isolates

Several tests were carried out to identify the bacterial isolates from the effluent, treated and untreated soil samples as described by Cheesbrough (2006). They were Gram staining, citrate utilization test, urease test, motility test, indole test, methyl red test, Voges-Proskauer test, coagulase test, oxidase test, spore test and sugar fermentation test.

2.11.1. Gram staining

A smear of the isolate on a clean slide was air-dried and heat fixed. The smear was flooded with crystal violet solution and rinsed off after 60 seconds with water. Subsequently, Lugol's iodine solution and acetone-alcohol were added to the smear and rinsed off after 60 seconds and 5 seconds respectively. The smear was then counterstained with Safranin and rinsed off after 10 seconds. The air-dried slide was examined under the microscope using x100 oil immersion objective lens. Purple color of the isolate indicated Gram positive while red color indicated Gram negative bacteria.

2.11.2. Citrate utilization test

Sterile Simmon's citrate agar was dispensed into different tubes and allowed to cool. The test isolate was inoculated on the agar and incubated at 37^0C for 24 hours. At the end of 24 hours, the change in color from green to blue indicated a positive result.

2.11.3. Urease test

Two hundred milliliters of water were sterilized and cooled before adding urea crystals (Christensen's modified urea broth) that was prepared according to the manufacturer's instruction. Equal volume of urea agar base was prepared and sterilized in a 250 ml conical flask and cooled properly. The urea solution was then added to the cooled urea agar base and mixed gently to give an amber color. Five milliliters were dispensed into test tubes for slants formation. The pure colonies of the test organisms were streaked on the slants and incubated at 37^0C for 24 hours. Positive results were shown by the production of pink color**.**

2.11.4. Motility test

It was performed using semi solid nutrient agar slant. Molten agar was dispensed into sterile test tubes required for the analysis. The tubes were sterilized and allowed to gel before making a straight stab of the organism using a sterile straight wire. A growth of the organism along the straight stab indicated non-motile organism while a growth away from the straight stab after 24hrs incubation indicated a positive result**.**

2.11.5. Indole test

Two tubes of peptone broth were obtained and one was inoculated with the isolates aseptically and the other broth left un-inoculated (a negative control) and incubated at 37^0C for 24 hours. The broths were removed from the incubator and 0.5ml kovac's reagent was added to each of them. The broths were allowed to stand for 15 minutes and red coloration on the top layer indicated a positive result**.**

2.11.6. Methyl-red test

The isolates were inoculated into the test tubes containing glucose phosphate peptone broth and incubated at 37^0C for 24 hours. Two drops of methyl red solution were added into the tubes after incubation and a red color appearance indicated a positive result while a yellow color indicated a negative result**.**

2.11.7. Voges-Proskauer test.

Glucose phosphate peptone broth was prepared according to the manufacturer's instructions and three milliliters of it were dispensed in sterile test tubes and pure culture of the test organisms were inoculated into the broth and incubated for 24 hours at 37^oC. 0.6 ml of 5% alpha naphthol was added, followed by 0.2 ml of 40% KOH. The tubes were shaken gently to expose the medium to atmospheric oxygen and allowed to stand undisturbed for 15 minutes. Positive tests were indicated by the development of a red color.

2.11.8. Coagulase test

A drop of normal saline was placed on each end of a slide and mixed with the isolate using a wire loop. Thick suspensions of each isolates were made. Two drops of plasma of human origin were added to one of the suspensions and mixed gently while no plasma was added to the second suspension which was used as the control. Clumping within 10 seconds indicated a positive result while absence of clumping indicated a negative**.**

2.11.9. Oxidase test

Sterile filter paper was placed in a sterile Petri-dish after which five drops of freshly prepared oxidase reagent were added. Using a sterile wire loop, smear of the organisms was made on the filter paper. The development of a blue-purple color within 10 seconds indicated a result.

2.11.10. Spore test.

The test sample was heat-fixed and flooded with 5% aqueous malachite green. The slide was steamed intermittently in a Bunsen burner after which it was washed under running water. It was counter stained with 0.5% aqueous Safranin for 15 seconds after which it was rinsed with water and drained. Presence of green color organism under the microscope indicated a positive result while pink color indicated a negative result**.**

2.11.11. Sugar fermentation test

The sugars tested were sucrose, lactose, glucose, mannitol, maltose and fructose. One gram of each of the sugars was weighed into different conical flasks (250mls). Seven hundred millilitres of peptone water were prepared according to the manufacturer's instruction and bromothymol blue indicator was added until the color changed to light blue. Equal volume was dispensed into the various conical flasks containing the different sugars and mixed gently by swirling. Ten millilitres of the resulting solution were dispensed into test tubes with Durham tubes, capped and autoclaved at 121^0C for 15 minutes. The tubes were allowed to cool at 25^0C and the isolates were inoculated and incubated at 37^0C for 48 hours. Positive results were shown by acid and gas production. The presence of gas in the Durham tubes indicated gas production while a change in color of the medium indicated acid production.

3. RESULTS

3.1. Bacterial quality of the untreated brewery effluent and the soil before and after pollution

The total bacterial count, total coliform count, fecal coliform count, total *Pseudomonas* count, total *Clostridium* count and total *Rhizobium* count in the effluent and soil samples are shown in figure 1. The total bacterial count of the effluent sample, untreated and treated soil samples were 5.2×10^3cfu/ml, 10.8×10^3cfu/g and 22.8×10^3cfu/g respectively. While zero fecal coliform count was recorded in all the samples, the total coliform counts were 0.5×10^3cfu/ml, 7.2×10^3cfu/g and 11.4×10^3cfu/g for the effluent sample, untreated and treated soil samples respectively. The highest *Clostridium* count, *Pseudomonas* count and *Rhizobium* count was recorded in the treated soil sample.

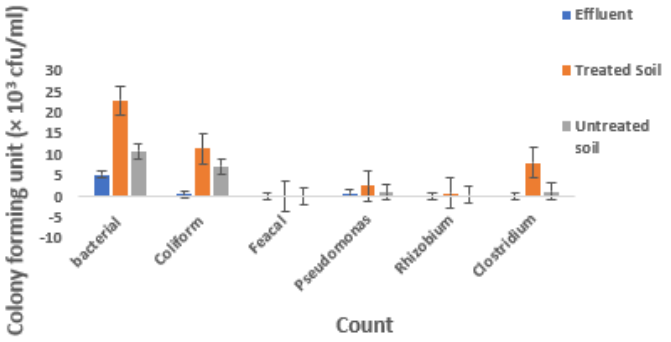

Figure 1: Bacterial quality of the brewery effluent and soil before and after treatment

3.2. Morphological and Biochemical Characterization of the Bacterial Isolates from the untreated brewery effluent

A total of 7 bacterial organisms belonging to four different genera *Pseudomonas, Streptococcus*, *Enterobacter* and *Serratia* were isolated from the effluent sample as represented in Table 1.

Table 1: Morphological and biochemical characteristics of the isolates from the brewery effluents

Isolate	1N^{w}	2N^{w}	3N^{w}	4N^{w}
Gram staining	-ve Rod	+ve Rod	-ve Rod	-ve Rod
Citrate	+	-	+	+
Urease	-	-	-	+
Motility	+	-	+	+
Indole	-	-	-	-
MR	-	-	-	-
VP	-	-	+	+
Coagulase	-	-	-	-
Oxidase	+	-	+	-
Spore	-	-	-	-
Sucrose	-	+	+	+
Lactose	-	+	+	-
Glucose	+	+	+	+
Mannitol	+	-	+	+
Maltose	+	+	+	+
Fructose	+	+	+	+

Key: 1N^{w} = *Pseudomonas* sp, 2N^{w}= *Streptococcus* sp, 3N^{w}= *Enterobacter* sp, 4N^{w}= *Serratia* sp

3.3. Morphological and Biochemical Characterization of the Bacterial Isolates from the soil samples before and after irrigation

A total of 27 bacterial organisms belonging to ten different genera *Pseudomonas, Bacillus*. *Azotobacter*, *Streptococcus*, *Staphylococcus*, *Rhizobium*, *Proteus*, *Enterobacter*, *Klebsiella* and *Serratia* were isolated from the untreated and treated soil samples as represented in Table 2.

3.4. Frequency of occurrence of the isolates in the brewery effluent and soil before and after treatment

Enterobacter sp was the highest occurring (42.9%) bacteria in the effluent sample; while *Bacillus* sp predominated in untreated soil and also occurred highest in the treated soil alongside *Streptococcus* sp and *Staphylococcus* sp (Table 3).

Table 2: Morphological and biochemical characteristics of the isolates from the soil (before and after irrigation)

Isolate	1N^{ut}	2N^{ut}	3N^{ut}	4N^{ut}	5N^{ut}	6N^{ut}	7N^{ut}	8N^{t}	9N^{ut}	10N^{t}
Gram staining	-ve Rod	+ve Rod	+ve Rod	+ve Rod	+ve cocci	-ve Rod	-ve Rod	-ve Rod	-ve Rod	-ve Rod
Citrate	+	+	+	-	+		-	+	+	+
Urease	-	-	+	-	+	+	+	-		+
Motility	+	+	+	-	-	-	-	+	-	+
Indole	-	-	+	-	-	-	-	-	+	-
MR	-	+	-	-	+	-	+	-	-	-
VP	-	-	-	-	+	-	-	+	+	+
Coagulase	-	-	-	-	+	-	-	-	-	-
Oxidase	+	+	+	-	-	+	-	+	-	-
Spore	-	+	-	-	-	-	-	-	-	-
Sucrose	-	-	+	+	+	-	-	+	+	+
Lactose	-	-	+	+	+	-	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	+	-	-	-	+	+	-	+	+	+
Maltose	+	+	-	+	+	+	-	+	+	+
Fructose	+	-	+	+	+	+	-	+	+	+

Key: Isolates with superscript 'ut' indicates organisms isolated from untreated soil while isolates with superscript 't' indicates organisms isolated from treated soil sample only.
1N^{ut} = *Pseudomonas* sp, 2N^{ut}= *Bacillus* sp, 3N^{ut}= *Azotobacter* sp, 4N^{ut}= *Streptococcus* sp 5N^{ut}= *Staphylococcus* sp, 6N^{ut}= *Rhizobium* sp, 7N^{ut}= *Proteus* sp, 8N^{t}= *Enterobacter* sp, 9N^{ut}= *Klebsiella* sp, 10N^{t}= *Serratia* sp

Table 3: Frequency of occurrence of the isolates in the brewery effluent and soil before and after treatment

Isolate	Number isolated			Frequency of occurrence (%)		
	Eff	Untr	treat	Eff	untr	treat
Pseudomonas sp.	1	1	2	14.3	7.7	14.3
Bacillus sp	0	4	2	0	30.8	14.3
Azotobacter sp	0	1	1	0	7.7	7.1
Streptococcus sp	2	1	2	28.6	7.7	14.3
Staphylococcus sp	0	1	2	0	7.7	14.3
Rhizobium sp	0	1	1	0	7.7	7.1
Proteus sp	0	3	1	0	23.1	7.1
Enterobacter sp	3	0	1	42.9	0	7.1
Klebsiella sp	0	1	1	0	7.7	7.1
Serratia sp	1	0	1	14.3	0	7.1
Total	**7**	**13**	**14**	**100**	**100**	**100**

Key: Eff = Effluent sample, untr = untreated soil sample, treat = polluted soil sample

4. Discussion

The present study determined the bacteriological quality of untreated brewery effluent and its effect on the bacteriological quality of agricultural soil. The bacterial analysis of the brewery effluent (figure 1) showed that total bacterial count and total coliform count were 5.2 × 10^{3}cfu/ml and 0.5× 10^{3}cfu/ml. No fecal coliform was recovered from the brewery effluent. The total bacterial count obtained in this study is in line with the work of Ikhajiagbe*et al.* (2014), who reported total bacterial count of 2.6 × 10^{3}cfu/ml during their work on microbial and

physiochemical quality of effluent water from a brewery in Benin City, Mid-Western Nigeria. Contrarily, Fakoredeet *al*. (2013), reported a high total bacterial count (2.9× 10^6cfu/ml) and total coliform count (9.0× 10^3cfu/ml) during their case study on the microbiological and physicochemical characterization of wastewater from a brewery in South-West Nigeria.

The soil sample treated with the brewery effluent had higher total bacterial count, total coliform, *Pseudomonas* count, Clostridial count and Rhizobial count than the untreated soil samples (Figure 1). The increased counts in the treated soil sample obtained in this study can be attributed to the impact of the brewery effluent used for the soil treatment; the bacterial load in the effluent added the load in the agricultural soil, and the contents of the effluent served as nutrient for growth for the organisms. The high bacterial count recorded in this study is in agreement with the report of Ike *et al*. (2019), who reported an increase in total heterotrophic bacterial count of soil when treated with different concentration of brewery sludge.

The bacteria genera, *Pseudomonas, Streptococcus*, *Staphylococcus*, *Enterobacter* and *Serratia,* (Table 1) isolated from the effluent have been isolated in brewery effluents by other researchers (Fakoredeet *al*., 2013; Ologbosereet *al*., 2016; Oljiraet *al.,* 2018).

Pseudomonas, Bacillus, *Azotobacter*, *Streptococcus*, *Staphylococcus*, *Rhizobium*, *Proteus* and *Klebsiella*were present in both treated and untreated soil samples (Table 2). However, *Enterobacter* and *Serratia* were only isolated in the treated soil sample, their presence in the treated soil sample result from the effluent used. This is in line with the work of Ike *et al* (2019) on impact of brewery wastewater sludge on microbial quality of agricultural soil.

Enterobacter sp occurred most in the effluent sample, *Bacillus* sp predominated in untreated soil while *Pseudomonas*, *Bacillus*, *Streptococcus* and *Staphylococcus* sp, occurred at the same frequency in the treated soil (Table.3). More bacterial organisms were isolated from the treated soil than the untreated soil and effluent sample. *Bacillus* sp. has been known to increase the uptake of cadmium and significantly increases root and shoot dry weight (Sheng, 2006). *Klebsiella, Rhizobium, Pseudomonas, Serratia* and *Azotobacter* are known as plant growth promoting bacteria (PGPB), they can enhance plant growth and protects plants from diseases and abiotic stresses through a wide variety of mechanisms (Rocheli*et al*., 2015). They also stimulate plant growth and decreases Cr6+ content (Rajkumar,2006).

All the individual organisms isolated has one or more beneficial roles to play in agricultural soil either to supply nutrients to crops, to stimulate plant growth through production of plant hormones, to control or inhibit the activities of plant pathogens, to improve soil structure or bioaccumulation or microbial leaching of inorganics (Ehrlich, 1997). The enrichment of the agricultural soil with brewery effluent enhanced the presence of bacterial organisms which are beneficial for plant growth.

5. Conclusion

The bacteriological analysis of Intafact Beverages Ltd. effluent was carried out and the result showed presence of several bacteria. The total bacteria count, total fecal count and total coliform count are within WHO (2006) recommended standard for irrigation water. The untreated brewery effluent influenced the bacteriological characteristic of the agricultural soil and the population of plant growth promoting bacteria was improved.

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