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Evaluation of two commercial inoculates and a probiotic food additive as seeds in BOD testing

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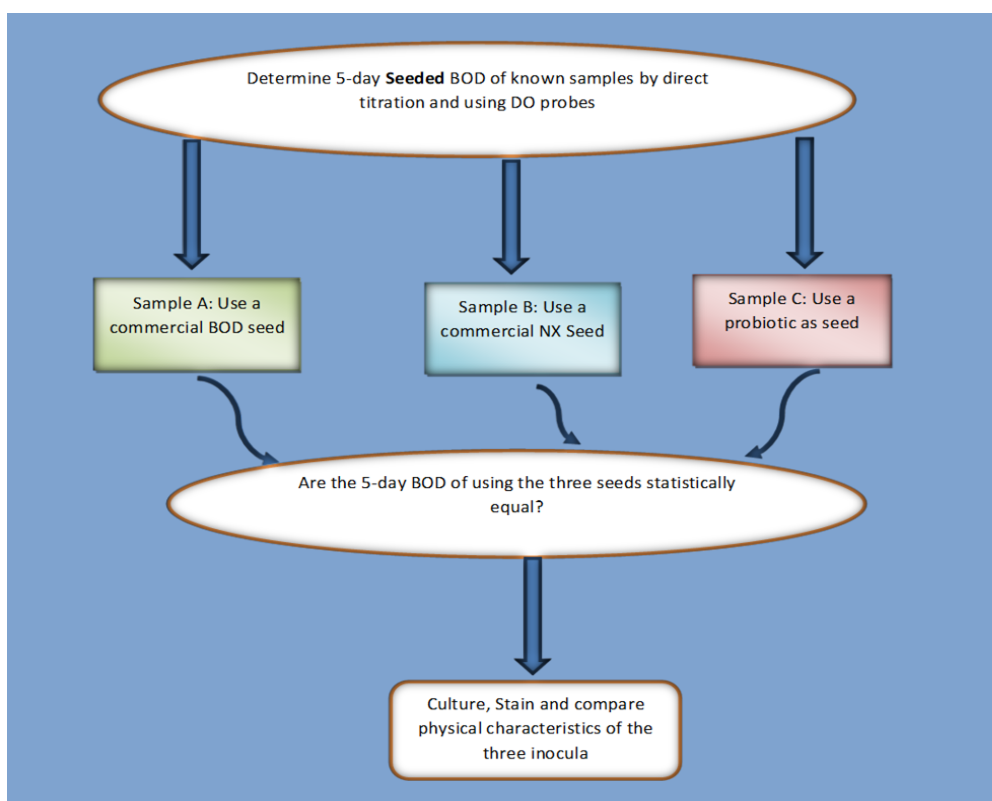
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Abstract

Five-day seeded Biochemical oxygen demand (BOD) tests using Glucose-Glutamic acid (GGA) substrate were carried out. The seeds used are a regular seed an NX seed and an over-the-counter food supplement commonly referred to as a probiotic. The purpose was to determine if seed type influenced 5-day BOD test results. The NX seed contained a chemical formulation designed to inhibit nitrification. Test results indicate that 5-day BOD varied significantly with each seed. Micrographs of stained samples prepared from cultures of the three seeds identified Gram-stain negative rods present in all the three inoculates.

Graphical abstract



Keywords: BOD test; Probiotic supplement; BOD seed; Gram stain.

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1. Introduction

The biochemical oxygen demand (BOD) test measures the organic content of water and wastes by the amount of oxygen consumed by microorganisms as they degrade a sample [1]. The standard test involves measuring the amount of dissolved oxygen in a diluted sample before and after a 5-day incubation period, with the difference being the oxygen consumed in the bio-reaction. The five-day BOD is the oxygen consumed during incubation, after factoring in dilution. The test is a bioassay involving mostly bacteria. In situations where the sample lacks microorganisms or has very few viable microorganisms, microbial are added to the mixture. Adding microbial to the BOD test is known as seeding. Microbial from wastewater treatment plants or commercially packaged microbes may be used. Some commercially available seeds have constituents that inhibit the growth of nitrifying microorganisms that increase oxygen consumption. A third inoculum sold over the counter as a nutritional additive and commonly referred to as probiotic, was also tested. WebMD [Www.webmd.com/digestive-disorders/features/what-are-probiotics, Last accessed 03/18/2019] describes probiotics as mainly bacteria and are members of the Bacillus family derived from a milk substrate culture.

The BOD of a sample is frequently the test of choice for measuring the organic content of water and wastewater. Total organic content (TOC), and the Chemical oxygen demand (COD) are ways for measuring the organic content in wastewater. Unlike the TOC and COD tests, the BOD test is a bioassay that simulates the biodegradation process in nature, thus microbial seeding may be required in situations where test samples are sterile or do not contain viable microbes.

For seeded BOD test, Equation 1, [1] is used.

$$\text{BOD} = \frac{(\text{DO}_i - \text{DO}_f)}{P} \quad (1)$$

Where;

BOD = biochemical oxygen demand of sample, (mg/L),

DO_i = initial dissolved oxygen concentration at day 0, (mg/L),

DO_f = final dissolved oxygen concentration at the day 5, (mg/L),

P = ratio of volume of sample used to total volume sample plus dilution water.

In the seeded BOD test, a second bottle containing dilution water and seed is prepared and incubated along with the seeded sample. This is necessary because the introduction of a seed into the sample bottle constitutes a change in the organic content of the sample. In the case of a seeded test Equation 2, [1] is used.

$$\text{BOD} = \frac{(\text{DO}_i - \text{DO}_f) - (\text{S}_i - \text{S}_f)F}{P} \quad (2)$$

Where;

BOD = biochemical oxygen demand of sample, (mg/L),

DO_i = initial dissolved oxygen concentration at day 0, (mg/L),

DO_f = final dissolved oxygen concentration at the day 5, (mg/L),

P = ratio of volume of sample used to total volume sample plus dilution water,

F = volume ratio of seed added to sample to seed added to the seed blank,

S_i = dissolved oxygen of seed blank at day 0, (mg/L),

S_f = dissolved oxygen of seed blank at day 5, (mg/L).

Several publications are available in the literature where seeds are evaluated or compared. Paixão et al. [2], compared two commercial BOD inocula (Bi-Chem and Biolen M112) in five-day BOD tests using standard glucose-glutamic acid and manometric titration methods. They also tested samples from treatment plants and concluded that both inocula had results that were comparable and not significantly different. Sharma et al. [3], using both synthetic and industrial samples and concluded that the consortium of microbes compared favorably with sewage seeds when tested for BOD. The isolated consortium of microbes was first immobilized on calcium alginate seeds. They recommend the use of the immobilized seeds in place of commercial seeds. Manoharan et al. [4], validated the use of a microbial consortium called "BODSEED" in BOD analysis of synthetic and other industrial material as a replacement for seed derived from sewage. Paixão et al. [5], investigated the potential of a soil inoculum and a pure culture of *Pseudomonas* sp. as alternatives to commercial BOD seeds. They tested samples of standard glucose-glutamic acid by the dilution and manometric methods for BOD testing, on substrates of standard glucose-glutamic acid solutions and concluded the alternatives were

potentially viable. Khan et al. [6], investigated three inocula and isolates from mixed liquor on two sources of water, secondary effluent, and raw surface water. The commercial seeds were BODSeed, Bi-Chem, and PolySeed. They reported that the commercial and indigenous inocula had similar BOD exertion trends; additionally, differences were insignificant on tests with surface water samples but were inconclusive for secondary effluent samples. BOD values in the mixed liquor inoculum tests were slightly higher and exertion kinetics were faster with the commercial and indigenous inocula.

Kumar et al. [7], reported the use of isolated microbes consisting of species of *Micrococcus*, *Staphylococcus* and *Pseudomonas* in BOD tests on pulp and paper wastes. They compared the isolates from domestic seed inoculate to commercially available seeds and concluded that they were similar. Furthermore, conventional and commercially available seeds performed poorly compared to the isolates. They recommend the use of special isolates in BOD tests involving hard to biodegrade wastes such as pulp and paper wastes. Mittal et al. [8], examined BOD exertion in dairy wastes, measuring microbial growth, with several seeds, by the optical density method. They concluded that metals such as nickel, cobalt, and zinc inhibited good microbes from human sources. Webber et al. [9], evaluated the impact of four factors (microbial strain, growth media composition, media strength, and microbial growth phase) on the BOD response profiles using Glucose-Glutamic Acid (GGA) and Organization for Economic Cooperation and Development (OECD) synthetic standard substrates. The investigators identified a microorganism that produced comparable BOD response profiles by two methods of BOD determination, the conventional bioassay and using BOD sensors. In a study, Dhall et al. [10], compared BOD to COD ratios of inoculum of an isolated consortium of microbes to the BOD to COD ratios of a commercially sold seed. The study yielded BOD to COD ratios of 0.5 – 0.6 for the commercial seed and 0.78 – 0.8 for the isolated bacteria strains. They concluded that BOD to COD ratios with the isolated consortium were more significant. Furthermore, the researchers [10] examined the effect of using a specific microbial consortium compared to commercial seeding material (BODSEED) on the BOD of dairy wastewater. They isolated bacterial strains from 5 different sources and screened by the conventional BOD method. The results were analyzed statistically by the t-test and indicated that the selected consortium had more significant BOD than the BODSEED. Jordan et al. [11], studied seeds sourced from wastewater treatment plant sludges using ferricyanide mediated (FM) five-day BOD and the traditional five-day BOD, concluding that sludge sourced from WWTP's can be seed for both types of tests.

The objective of the study was to compare five-day BOD values obtained with three inocula as seeds. The inocula were, a commercially sold regular BOD seed, and a denitrifying seed, and a food additive commonly referred to as a probiotic.

2. Material and methods

PolySeed, PolySeed NX, and a probiotic, manufactured by the Spring Valley company, were the inoculates. PolySeed and PolySeed NX are blends of broad-spectrum bacteria commercially sold as BOD seeds. PolySeed NX inhibits nitrification in samples. The commercial seeds prepared following the manufacturer's directions; the contents of a capsule were placed in 500 ml of dilution water and aerated for an hour and allowed to settle for 5 to 15 mins. The supernatant was decanted into a 500-ml beaker and ready for use.

The Wal-Mart corporation retails the Spring Valley probiotic used in the study. Probiotics are live bacteria that promote human health. The manufacturer of Spring Valley probiotic claims that each capsule has over 4 billion active organisms. Other ingredients in probiotic capsules include Maltodextrin, Starch, Hypromellose, Magnesium stearate, Titanium dioxide, and Ascorbic acid. The preparation of the probiotic for use as a seed followed a similar process as described for the commercial seeds.

The BOD tests used standard 300 ml BOD glass bottles with grounded stoppers and BOD nutrient buffer capsules for dilution. In each 300 ml bottle, a buffer capsule broken into the bottle and topped with distilled water. Standard GCA, marketed by the Hach corporation, was the substrate. The researchers prepared BOD test bottles according to the Water Environment Federation [12], recommendations. Dissolved oxygen levels were measured by the modified Winkler titration procedure as described in Standard Methods for the Examination of Water and Wastewater [12], and by using a DO meter, model YSI 550 (YSI Inc).

3. Results

3.1. BOD tests

Table 1 and Table 2 show the five-day BOD test results, with means and standard deviations, by the oxygen probe and by the Winkler titration methods. BOD's were graphically examined for conformity to a Normal distribution in Figures

1-6, using the Minitab software [13]. Although non-quantitative, the figures do show approximate conformity with the Normal distribution. Also, from Table 3, BOD values by the two methods of oxygen measurement are not statistically equal at the 95% level of significance ($\alpha = 0.05$). The Winkler titration technique is the standard, and so the authors chose BOD results from the Winkler titration procedure for further analyses.

Figure 7 depicts the spread of standard deviations of BOD's with the three inoculates. The statistical comparison of BOD values with the three seeds are depicted in Table 3. The results indicate that the BOD's are not equal based on the t-test ($\alpha = 0.05$).

Table 1 Results BOD₅ of tests using three microbial Inocula and the DO probe.

	Regular Seed	NX Seed	Probiotic
Sample #	BOD_{5,20} (mg/L)	BOD_{5,20} (mg/L)	BOD_{5,20} (mg/L)
1	175	188	204
2	177	184	210
3	174	181	220
4	179	177	212
5	168	192	200
6	173	196	217
7	174	186	211
8	178	191	213
9	170	183	206
10	175	185	215
Mean	174	186	211
Std. Dev.	3.40	5.62	6.09

Table 2 Results of BOD₅ tests using three microbial Inocula and the Winkler Method.

	Regular Seed	NX Seed	Probiotic
Sample #	BOD_{5,20} (mg/L)	BOD_{5,20} (mg/L)	BOD_{5,20} (mg/L)
1	186	210	218
2	189	204	226
3	184	199	227
4	187	206	219
5	193	209	218
6	188	213	222
7	186	204	223
8	187	210	219
9	185	205	220
10	192	208	225
Mean	188	207	222
Std. Dev.	2.91	4.02	3.40

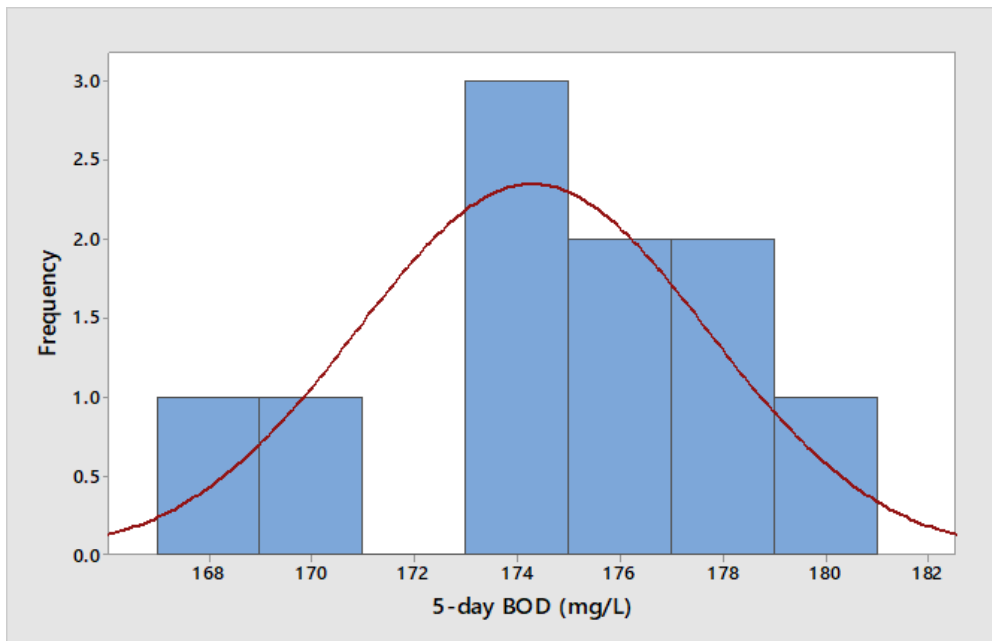


Figure 1 Frequency curve using five-day BOD of PolySeed with Normal curve (Oxygen Probe)

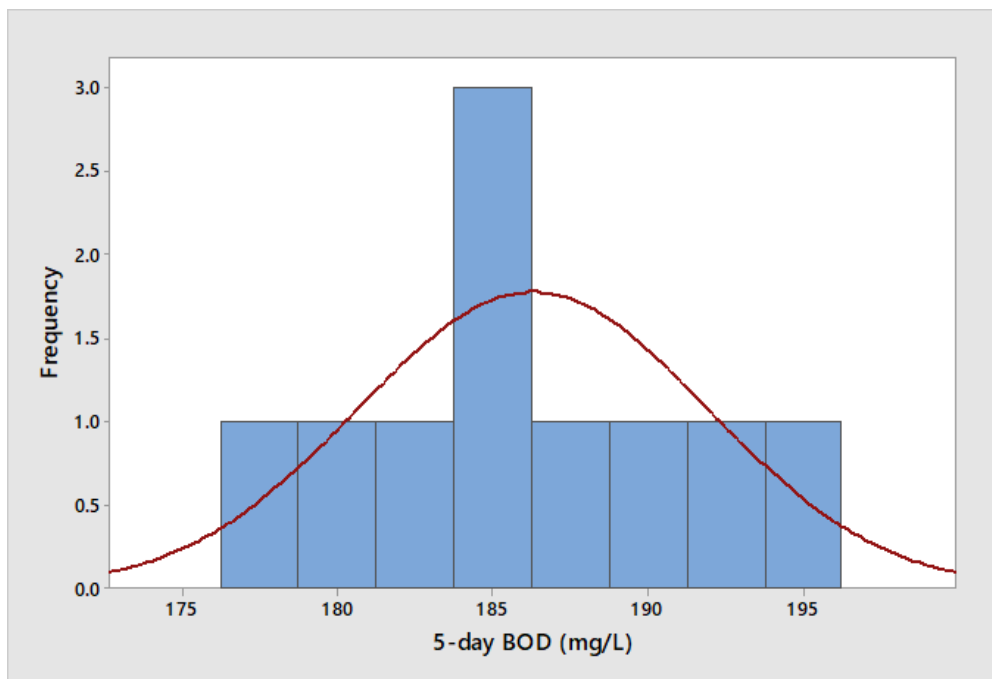


Figure 2 Frequency curve using five-day BOD of NX Seed with Normal curve (Oxygen Probe)

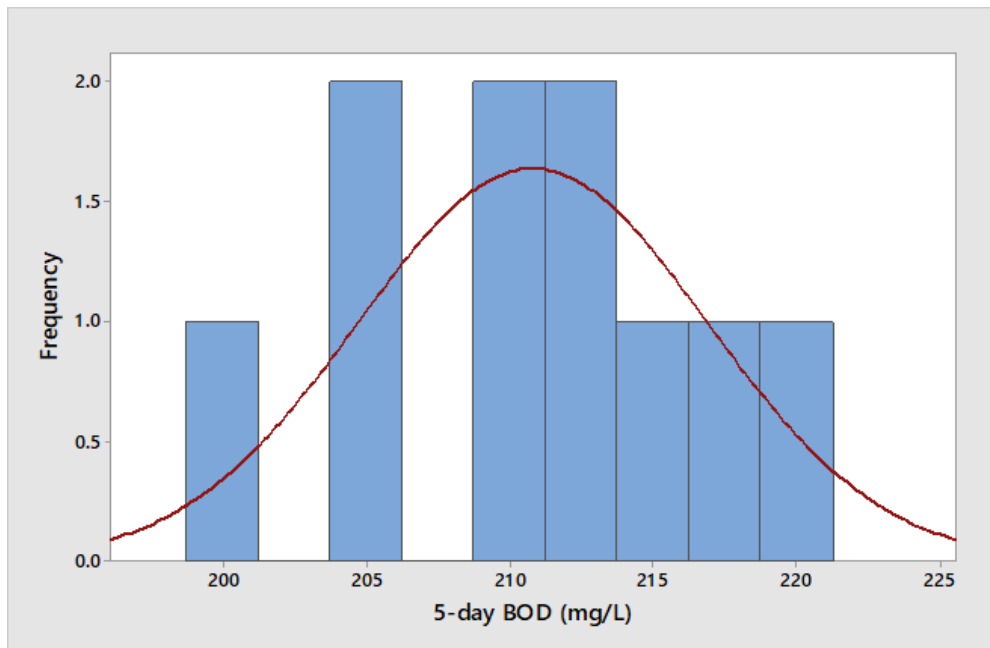


Figure 3 Frequency curve using five-day probiotic seed with Normal curve (Oxygen Probe)

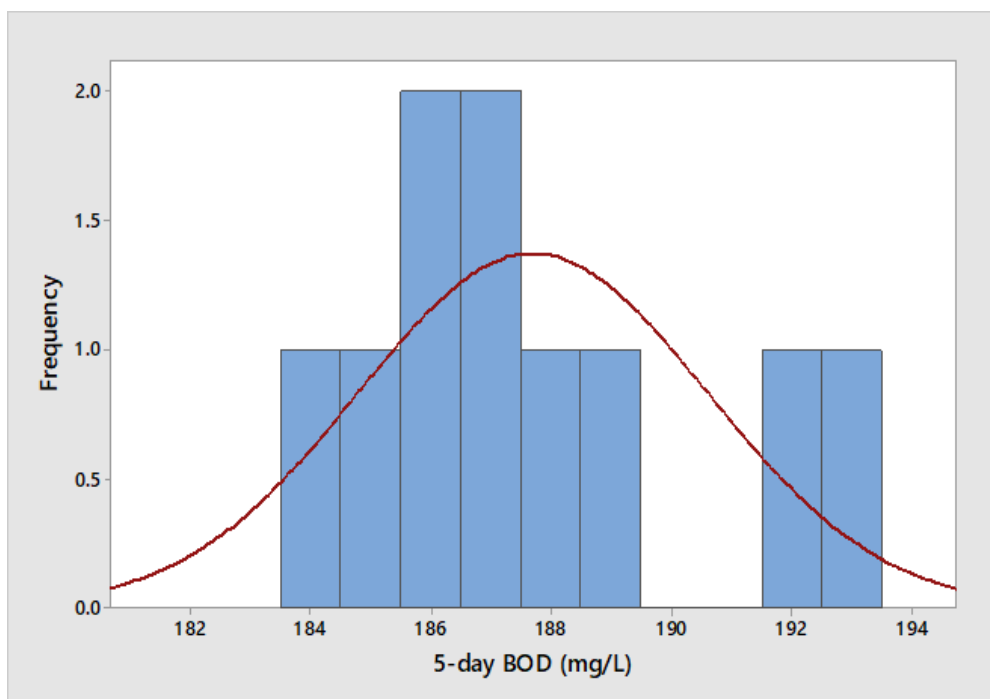


Figure 4 Frequency curve using five-day BOD of PolySeed with Normal curve (Winkler method).

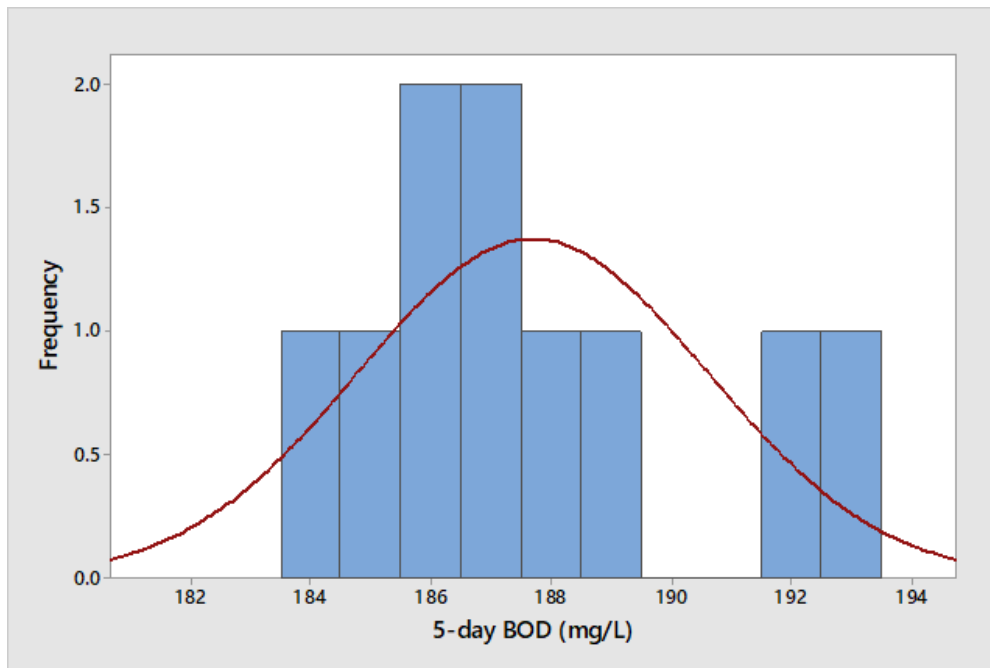


Figure 5 Frequency curve of five-day BOD using NX seed with Normal curve (Winkler method)

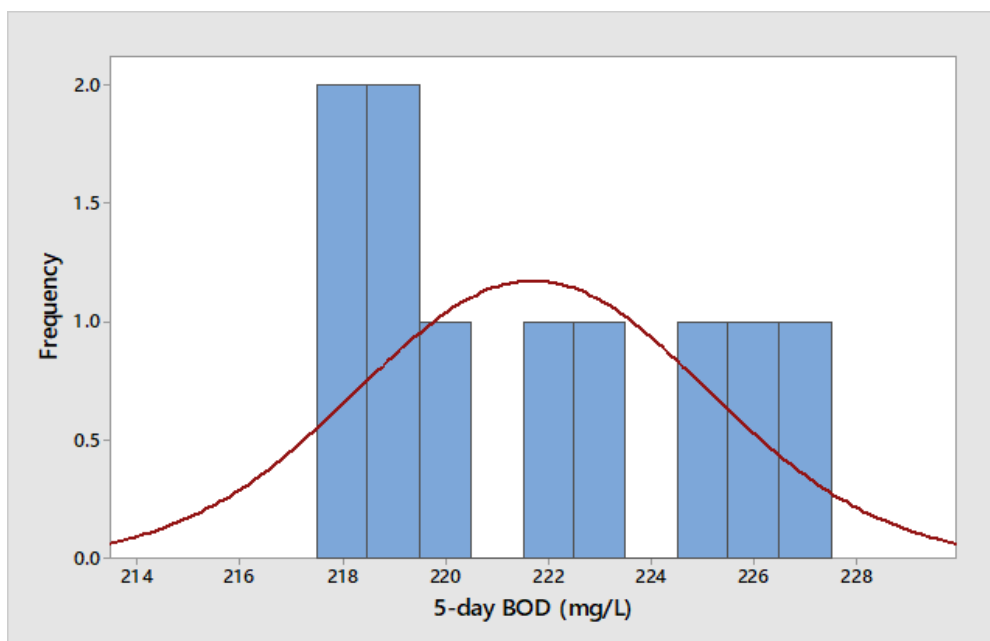


Figure 6 Frequency curve of five-day BOD using probiotic seed with Normal curve (Winkler method)

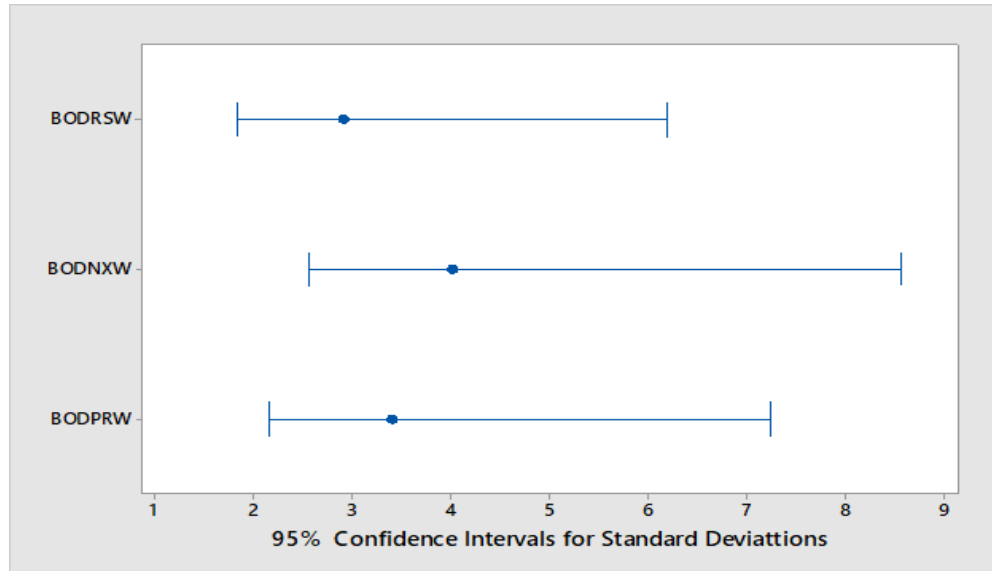


Figure 7 Standard deviations of 5-day BOD of the three inocula with the 95% confidence band

Table 3 T-test comparison of 5-day BOD values using a DO probe to the Winkler method ($\alpha=0.05$)

Test	t statistic	P value (%)	Conclusion
BODRSW > BODRSP	9.4700	0.00	Yes
BODRSW < BODRSP	9.4700	1.00	No
BODNXW > BODNXP	9.3819	0.00	Yes
BODRSW < BODNXP	9.3819	1.00	No
BODPRW > BODPRP	4.9426	0.00	Yes
BODPRW < BODPRP	4.9426	1.00	No

3.2. Gram stains

The team prepared samples for the Gram-Stain procedure in a manner to a preparation for BOD tests. After incubation at 20°C for 48 hours, the samples were stained using the Gram Stain procedure as follows; a millimeter of the sample was placed on a slide, air-dried and then flame dried. It was subsequently cooled and dyed. The flame-dried samples were stained using a three-stage procedure with the addition of a few drops of Hucker's Crystal Violet on the, followed by Iodine solution, and then discolored with an acetone-alcohol solution. Finally, three drops of Safranin were applied to the sample to complete the process.

3.3. Micrographs

Gram-positive bacteria will maintain the purple color of the dye, however, Gram-negative bacteria with thinner cell walls, assume the red-brown color of the Safranin. The stained slides are shown in Figures 8, 9, and 10, photographed at a 1000 magnification. The bacteria in the micrographs are rod-shaped and Gram-negative in every case.

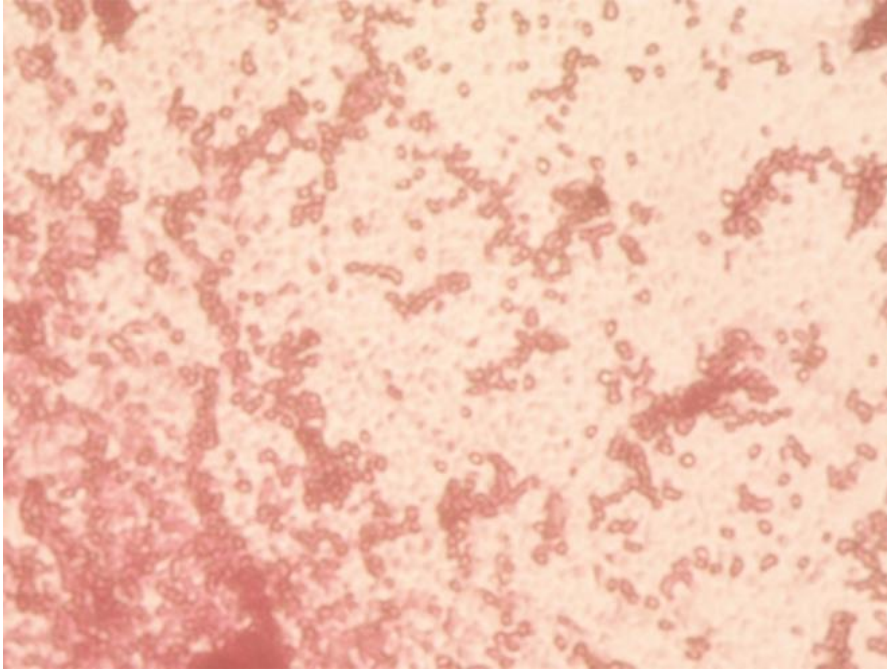


Figure 8 Cultured PolySeed micrograph at 1000 magnification

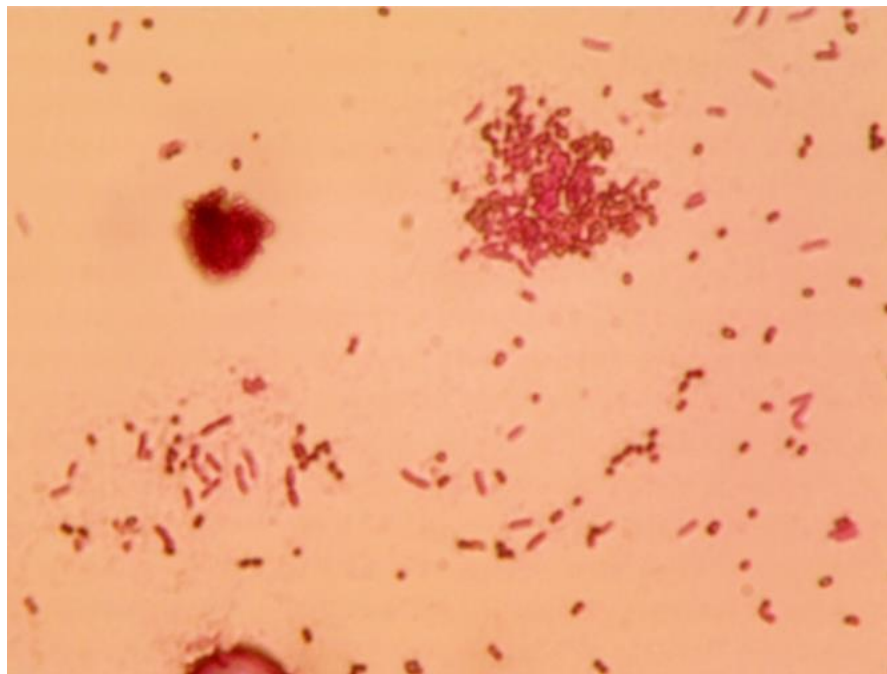


Figure 9 Cultured NX seed micrograph at 1000 magnification

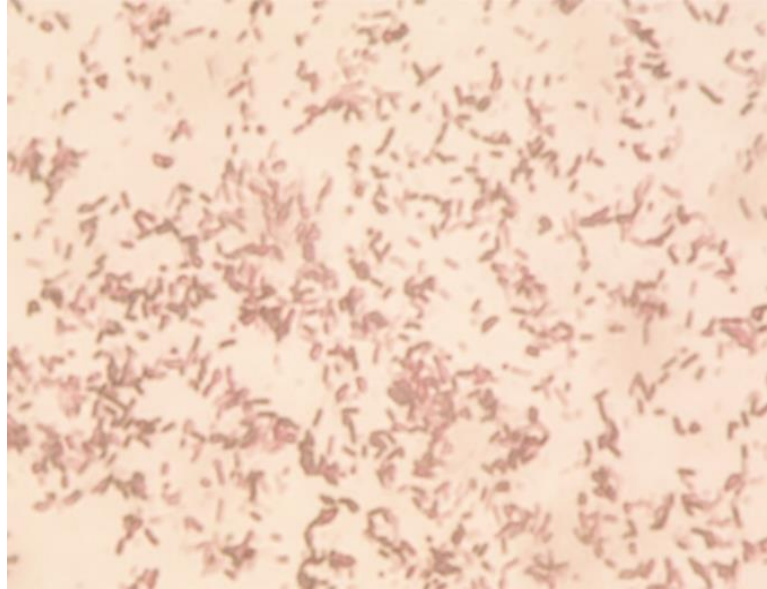


Figure 10 Cultured probiotic seed micrograph at 1000 magnification

4. Conclusion

The authors investigated the performance of two commercially marketed BOD seeds, and the potential use of an over-the-counter food additive probiotic as BOD seeds. Glutamic acid was the substrate for the BOD tests. The conclusion is that seed-type in seeded BOD tests can influence test results because 5-day BOD's from the three seeds were statistically unequal at the 95% confidence level. The study also indicated a potential for future use of probiotics as a seed in BOD tests. They identified Gram-stain-negative and rod-shaped bacteria from prepared micrographs. The authors recommend further testing of probiotics' potential for use as BOD seeds.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that the submitted work was not carried out in the presence of personal, professional or financial relationships that could be potentially construed as a conflict of interest.

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