Enhancing Wastewater Treatment by Commercial and Native Microbial Inocula with Factorial Design

Sahar El-Shatoury[1], Amro El-Baz[2*], Mahmoud Abdel daiem[2] and Diaa El-Monayeri[2]

[1] Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt
[2] Environmental Engineering Department, Faculty of Engineering, Zagazig University, 44519, Zagazig, Egypt
* elbaz50@hotmail.com

Abstract: Batch experiments were conducted on organic removal from wastewater by a commercial and native microbial inocula. The interaction effects of organic loading and excess aeration on the removal process was investigated using factorial design experiment. Biochemical oxygen demand (BOD) removal was significantly affected by the interactions of microbial inocula with aeration; their interactions could collectively explain 67.3% of the results. The effect of native inoculum on BOD removal was more evident under low aerated conditions and high effective microorganisms (EM) inoculum Ammonia removal was significant under aerated conditions and low EM density, reaching to mean removal values of 78% after 5 hrs. Compared to EM, the individual strains of the *Actinomycetes consortium* showed higher enzyme efficiency in degrading carbohydrate and lipid compounds, while less activity to degrade protein compounds. Although, both inocula achieved good and comparable suspended solid removal values, the native consortium showed better inhibition of faecal coliforms. It is suggested that combination of commercial and native inocula may enhance wastewater treatment where both groups cooperate via complementary metabolic pathway.

[Sahar El-Shatoury, Amro El-Baz, Mahmoud Abdel daiem and Diaa El-Monayeri. **Enhancing Wastewater Treatment by Commercial and Native Microbial Inocula with Factorial Design**. *Life Sci. J* 2014; 11(7):736-742] (ISSN: 1097-8135). http://www.lifesciencesite.com. 108

Keywords: Effective microorganisms – *Actinomycetesconsortium*–Two-level factorial design – Enzymatic activities - Municipal wastewater

1.Introduction

Upgrading of wastewater treatment systems to attain the increasing stringent legislation on environmental standards requires an improvement in organic pollutants removal efficiency of such systems. A viable approach is bioaugmentation with external microorganisms (Lendvay*et al.,* 2003, El Fantroussi andAgathos, 2005), where several types of commercial microbial products (inocula) exist in the market. An example is the effective microorganisms (EM), which is amulti-culture of coexisting anaerobic and aerobic beneficial microorganisms comprising lactic acid bacteria, photosynthetic bacteria, yeasts and actinomycetes (Lee and Cho, 2010, Ahn*et al.,* 2014). It was hypothesized that EM in wastewater treatment is beneficial in reducing sludge volumes and that EM might reduce the population of pathogenic bacteria through competitive exclusion (SzymanskiandPatterson, 2003).

Bioaugmentation with native 'indigenous' microorganisms may improve treatment, compared to bioaugmentations using commercial inocula (Loperena*et al.,*2009; Cosgrove*et al.,*2010). This is due to thevariable conditions that exist within wastewater which represent an ecological barrier for successful bioremediation performance of commercial inocula. It is becoming more evidenced that, the best way to overcome such ecological barriers is to look for microorganisms from the same ecological niche as the polluted area (El Fantroussi andAgathos, 2005).

In biological wastewater treatment, organic compounds are oxidized by microorganisms for energy production and multiplication. If there are oxidized nitrogen forms in wastewater, organic compounds are also used for their reduction (Zielińska and Wojnowska-Baryła, 2004). Aerobic conditions is required forautotrophs to oxidize ammonium nitrogen to nitrate (nitrification) in two subsequent steps, mainly by *Nitrosomonas* and *Nitrobacter* species. Nitrate formation is usually considered as the rate-limiting step in the whole nitrification process. Nitrate is then converted to molecular nitrogen by heterotrophic bacteria under anoxic condition during denitrification step (Khin and Annachhatre, 2004). Therefore, carbon and nitrogen removal requires both aerobic and anoxic conditions sequentially or simultaneously. There have been limited reports on using the statistical methods to investigate the interactions among microbial inocula in wastewater treatment (Chen *et al.,*2009, Srinivasan and Viraraghavan, 2010). In this study, a factorial design experiments was applied to evaluate the interactions of inoculated commercial and native microbial consortia with aeration and nutrient biostimulation for enhancing wastewater treatment. It is expected that, these experiments will help in defining the relative importance of microbial inocula for bioaugmentation in wastewater treatment.

2. Materials and Methods

2.1.Microbial inocula activation

The commercial inoculum, EM, was obtained from the Ministry of Agriculture – Egypt and activated according to manufacturer's instructions (Lee and Cho, 2010). Briefly, 100 ml EM were added to 100 ml molasses and 1.8 L distilled water. The mixture was kept in a 3 L closed tank and stored at room temperature for 7 days prior to application.

The native inoculum consisted of six strains of actinomycetes: *Micromonosporacarbonaceae 19*, *M. chalcea 32*, *M. aurantiaca 56*, *M. inositola 37*, *M. sp 62* and *Kineosporasp. 66*. These strains were isolated from industrial wastewater (El-Shatoury*et al.,*2004). Each strain was activated in 100 ml yeast extract dextrose broth and incubated at 28°C, shacked at 100 rpm for 5 days; the activated mycelia were harvested in the late exponential phase. The mycelia collected after centrifugation (6000 rpm for 10 min) were washed in phosphate buffer, resuspended in the buffer to give an initial optical density at 600nm of 0.20 ±0.01 and combined as a native inoculum prior to use in the subsequent experiments.

2.2.Characteristics of wastewater

Wastewater was collected from an activated sludge system receiving municipal wastewater. Its characteristics were analysed according to APHA 22nd Ed. (Rice, *et al.,*2012).Nitrate and ammonia were estimated using WinLab® Photometer LF 2400 (based on colorimetric methods SM 4500-NO_3^- and SM 4500-NH_3F, respectively); readings were recorded according to the manufacturer's instructions. BOD was determined using 5-day BOD test (SM 5210B). TSS was measured by the filtration (SM2540D).Dissolved oxygen and pH were measured using WinLab probes; Faecal coliform was counted on ENDO agar after incubation at 44.5°C for 24 hours(SM 9222D). The wastewater characteristics in this study are shown in Table(1).

Table 1.Physico-chemical and microbiological characteristics of inlet wastewater

Parameter	Average value
Ammonium nitrogen (NH_4), mg/l	0.6 – 1.35
Nitrate nitrogen (NO_3), mg/l	1.9 - 2.14
Biochemical oxygen demand (BOD), mg/l	300±27
Total suspended solids (TSS), mg/l	744±45
Dissolved oxygen (DO), mg/l	0.9±0.4
pH value	7.0±35
Faecal coliform (FC) *10^3 CFU / ml	6±0.9

2.3. Performance of EM inoculum *vs* native inoculum in wastewater

Batch experiment was performed to investigate the improvement in wastewater quality by augmentation with the commercial and native consortia. Experiment was performed in 2-liter beakers, containing 1 L wastewater samples from the activated sludge system previously described (section 2.2). The wastewater was inoculated with the investigated inocula (1:1000 w/v ratio); aeration was adjusted at 0.6 l/min using air pump X-8. Experiments were conducted in duplicates and un-inoculated controls were included. Samples were taken at2-hoursintervals and analyzed for total suspended solids and faecal coliform bacteria as indicators of the removal of organic and microbial pollutants, respectively, from wastewater.

2.4. Enzymatic activities of the native inoculum

Enzymatic activities of individual strains of actinomycetes were measured as activity index (diameter of clear zone / diameter of growth), according to (Williams, and Wellington, 1982) as follows:

Amylase production:

The strains were inoculated in Starch Agar medium plates, incubated for 14 days at 28°C, then covered by Gram's iodine solution, which allowed the visualization of clear halos around the colonies that produced amylase.

Lipase production:

The strains were grown on Tributyrin agar plates at 28ºC for 7days. The clear zone around the growth indicated the lipase production.

CMC-ase production:

The strains were inoculated into a basal medium containing carboxymethyl cellulose (CMC) and incubated for 9 days at 28°C. To detect hydrolysis zone, the plates were flooded with 1% (w/v) aqueous solution of hexadecyltrimethyl ammonium bromide to precipitate undegraded CMC.

Protease production:

The media of Frazier's gelatin agar was inoculated with the strains. After incubation at 28ºC for 14 day, the plates were covered with Frazier's revealers (distilled water 100 ml, HCl 20.0 ml and mercury dichloride 15.0 g). The proteolytic activity was indicated by clear halo around the actinomycetes growth.

2.5.Statistical methods

A two-level factorial design of experiments was used to investigate the removal of organic pollutants from wastewater by the commercial and native inocula. The organics removal caused by four main effects: (A) EM density, (B) organic load, (C) excess aeration, (D) native inoculum and their interactions were assessed by *T* statistics and analysis of variance (ANOVA). Removal efficiency of biochemical oxygen demand (BOD), nitrate (NO_3) and ammonia (NH_4) were measured as the responses. A total of 16 experiments were generated by a two level factorial design (2^4) using the statistical software MINITAB release 15.

EM was applied to wastewater at low (L) and high (H) density, expressed as F/M ratio 0.05 and 0.005 respectively (mg of BOD_5/mg of EM). The native inoculum was added at 1:1000 v/v (coded 1 and 0 for addition and absence, respectively) (Loperena, *et al.,* 2006). Aeration was adjusted at 0.6 l/min using air pump X-8. High organic load was achieved by incorporation of peptone (expressed as 1200 BOD mg/l) to wastewater (Holler, and Trösch, 2001). Experiments were performed in 2-liter size beakers, each contained 1-litre wastewater sample. Experiments were conducted in duplicates; after five hours, duplicate samples were collected for BOD, ammonia and nitrate analysis as described above. Detailed experimental design is shown in Table 2.

Table 2. Two level factorial design for investigating effects of EM density, organic load, excess aeration and native inoculum on wastewater treatment.

Std. Order	Run Order	Factors				Response(removal %)		
		Commercial EM	Organic Load (BOD mg/l)	Aeration	Native inoculum	BOD	NO_3	NH_4
8	1	L	1200	1	0	77.6	57.0	88.8
6	2	L	300	1	0	58.4	0	51. 3
5	3	H	300	1	0	46.4	0	0
16	4	L	1200	1	1	0.0	6.4	83.8
2	5	L	300	0	0	5.7	0	0
12	6	L	1200	0	1	3.7	0	0
4	7	L	1200	0	0	2.9	0	0
10	8	L	300	0	1	10.7	0	0
7	9	H	1200	1	0	8.1	0	0
15	10	H	1200	1	1	2.1	27.0	0
1	11	H	300	0	0	0.0	21.2	0
3	12	H	1200	0	0	5.1	0	0
9	13	H	300	0	1	64.8	0	23.8
14	14	L	300	1	1	2.7	0	81. 1
11	15	H	1200	0	1	73.7	2.1	85.7
13	16	H	300	1	1	7.5	13.0	0

3. Results and Discussion

3.1. Influence of *Actinomycetes consortium*on the biotreatment

The efficiency of commercial EM was compared with that of the native actinomycetes consortium in batch experiments. Both inocula were efficient in lowering suspended solids of wastewater and were capable to produce hydrolytic enzymes. Compared to EM, the individual strains of the *Actinomycetes consortium* showed higher enzyme efficiency in degrading carbohydrate and lipid compounds, while less activity to degrade protein compounds (Table 3).This was in agreement with previous investigations which reported positive impact of both commercial and native inocula in enhancing wastewater quality (Szymanski and Patterson, 2003, Loperena, *et al.,*2006).

Table3. Average enzymatic activity indices of amylase, protease, lipase and carboxymethyl cellulase(CMCase) for the native actinomycete inoculum, compared to commercial EM.

Enzyme	Native inoculum(6 actinomycetestrains)						Commercial inoculum (EM)*
	***M.* 19**	***M.* 32**	***M.* 56**	***M.* 37**	***M.* 62**	***K.* 66**	
Amylase	2	1.9	3.1	2	1.7	3.5	6.6
Caboxymethylcellulase	na	na	1.8	na	na	2	na
Lipase	na	na	6.6	na	na	3.5	na
Protease	na	na	na	na	na	na	3.53

* Results for EM from El Karamany*et al.,* 2013; na: No activity.

Nevertheless, the efficiency of actinomycetes in faecal coliform removal was significantly higher (p= 0.02) than the commercial EM inoculum (Figure,1). The actinomycetes had an advantage to reduce the faecal coliform counts, compared to EM. Effectiveness of actinomycetes in coliform reduction is attributed to their characteristic metabolic activities that make them an efficient inhibitor for microbial pathogens and the producer of over 85% of the antimicrobial compounds in use today (Baltz, 2008). Actinomycetes from open-pond wastewater system were reported to inhibit faecal indicators and pathogenic bacteria (Saenna*et al.,* 2011). A comparison study on kinetic properties of commercial and native inocula for wastewater treatment has also indicated better acclimatization and more efficacy of the native inoculum (Loperena*et al.,* 2006).

3.2. Effect of commercial and native inocula on wastewater treatment

A two-level factorial design of experiments was conducted to identify the factors that can enhance the removal of organics in wastewater. Removal percentages of BOD, nitrate and ammonia were measured after 5 hrs. Two-level experimental design is effective to evaluate the whole set of main effects as well as interaction effects. The main effects and the lower-order interactions, however, are usually the most significant terms (Montgomery *et al.,* 2007).

Figure (2) is a Pareto chart of the standardized effects and their first-order interactions for organics removal. The effects/interactions that lie before the line are negligible, whereas significant ones are those crossing the line with values higher than 2.57 ($p \leq 0.05$).

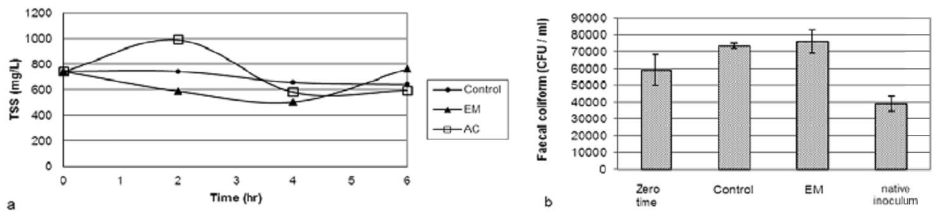


Figure 1. Effect of commercial EM and native actinomycetes inocula on total suspended solids (a) and faecal coliforms reduction after 6 hours (b) in batch experiment.

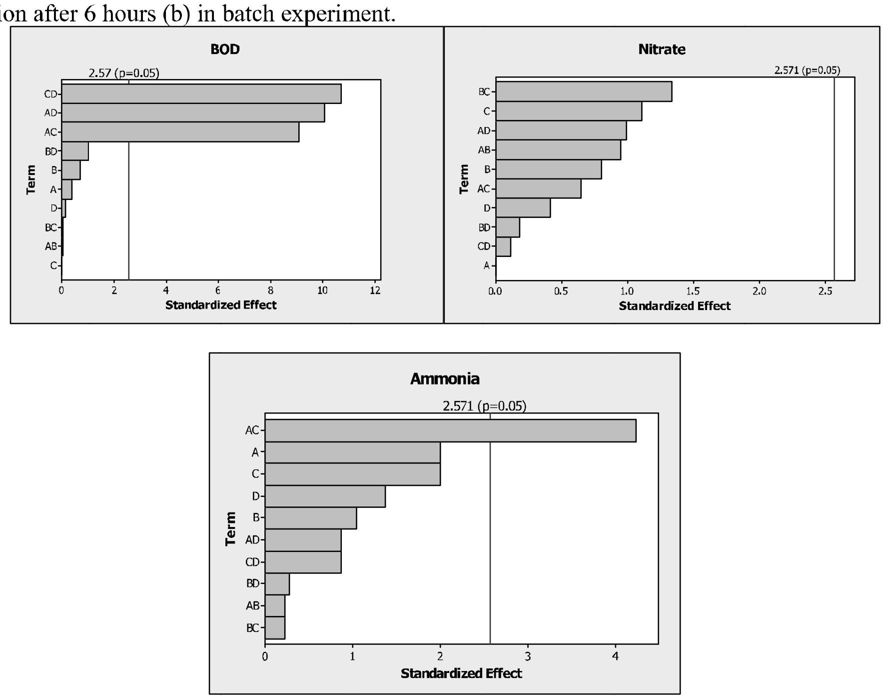


Figure 2. Pareto chart of the standardized effects for removal of BOD, nitrate and ammonia from wastewater. Factors: (A) concentration of commercial EM inoculum, (B) organic load, (C) aeration and (D) presence of native inoculum.

Under the investigated conditions, only BOD and ammonia were significantly affected. Our results indicated that the interaction between factors was significantly more effective than the individual factors in organics removal. For example, BOD removal was significantly affected by the interactions of aeration and microbial inocula; their interactions could collectively explain 67.3% of the results as illustrated in Table 4.

The effect of native inoculum on BOD removal was more evident under low aerated conditions and high EM inoculum (Figs 3a,b), indicating that coexistence of *EM and native actinomycetes* produced a synergistic effect in degrading organics. This is in agreement with Sheng *et al.*(2008) who reported that EM is predominantly capable of degrading organic material under low aerobic conditions.

Table 4. Estimated effects and coefficients for major factors affecting BOD and ammonia removal

Factors	**Effect**	**t- Statistics**	***P*-value**
BOD removal			
Constant		12.57	0.000
EMdensity (A)	-1.30	-0.40	0.704
Organic load (B)	2.38	0.74	0.495
Excess aeration (C)	0.03	0.01	0.992
Native inoculum (D)	-0.42	-0.13	0.901
A* B	-0.21	-0.06	0.952
A * C	**29.38**	9.08	**0.000**
A * D	**-32.56**	-10.07	**0.000**
B * C	0.23	0.07	0.947
B * D	-3.40	-1.05	0.341
C * D	**-34.69**	-10.72	**0.000**
NH4 removal			
Constant		4.23	0.008
EMdensity (A)	24.432	2.00	0.102
Organic load (B)	12.773	1.04	0.345
Excess Aeration (C)	24.432	2.00	0.102
Native inoculum (D)	16.795	1.37	0.228
A* B	-2.705	-0.22	0.834
A * C	**51.818**	4.23	**0.008**
A * D	-10.591	-0.87	0.426
B * C	-2.705	-0.22	0.834
B * D	3.386	0.28	0.793
C * D	-10.591	-0.87	0.426

Values in bold indicate significance at $p< 0.05$.

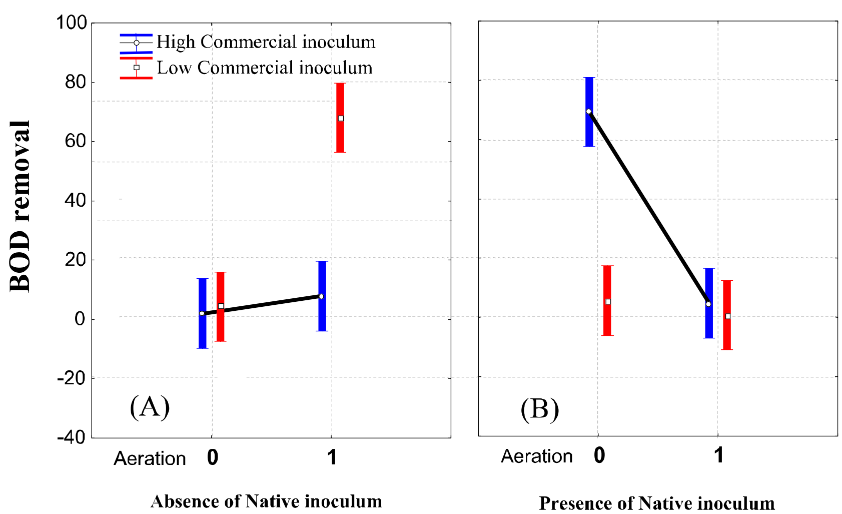
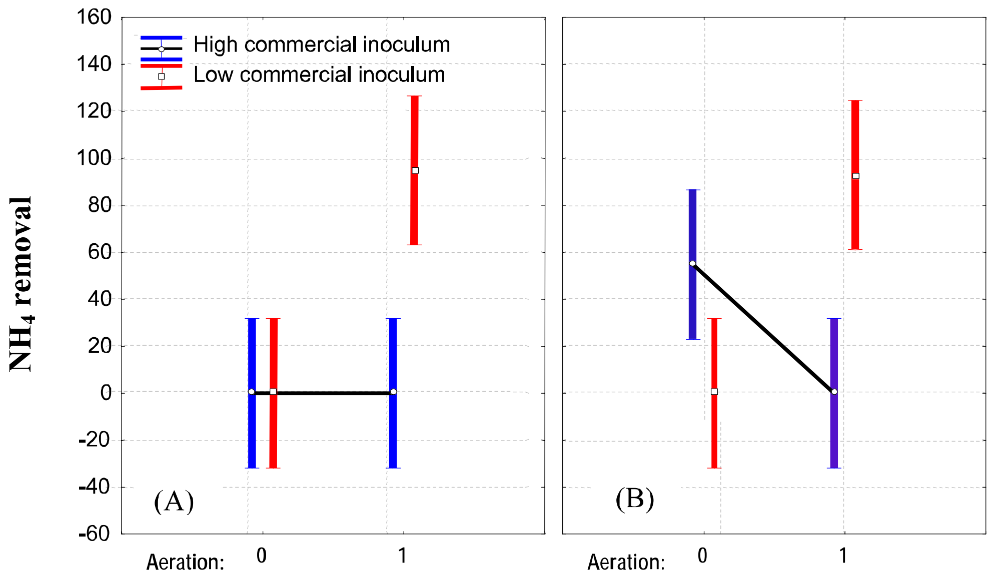


Figure 3. Interactions of aeration, commercial inoculum and native inoculum for BOD removal from wastewater

The applied native inoculum mainly consisted of *Micromonospora* strains which preferably shows degradation abilities under low aerobic conditions. Our results are also consistent with that of de Menezes*et al.* (2012) who used genetic analysis to offer strong evidence for active *Micromonospora* populations with significant role in degrading organics under microaerophilic conditions in lakes.

However, the native inoculum did not show significant contribution to ammonia removal (Table 4, and Figure 4). Only commercial inoculum and aeration showed a significant negative interaction (p= 0.008); the estimated effect of this interaction could explain 51.81% of ammonia removal results. The interaction plot (Figure 4a) showed that, the influence of EM on ammonia removal is significant only under excess aeration, reaching to mean removal values of 78%. While, the presence of native inoculum under the same mentioned conditions didn't significantly enhance ammonia removal (Figure 4b). This result is consistent with previous results for an EM-immobilized system treating synthetic municipal wastewater (Lee and Cho, 2010), where high ratio of aeration time to anoxic time increased the removal efficiency of total nitrogen. It is explained that more aeration is required by the autotrophic species in EM for oxidizing ammonium nitrogen to nitrate.



Absence of Native inoculum | Presence of Native inoculum

Figure 4. Interactions of aeration and commercial inoculum for ammonia removal from wastewater.

3.3 Conclusion

In conclusion, commercial and native inocula synergistically degrade organic pollutants in wastewater. Their syntrophic interactions could be indicated from the different enzyme profiling.The native actinomycetes inoculum showed significant and better performance for inhibiting faecal coliform. From the d*999etailed investigation of factors affecting bioaugmentation, biological nature of the inocula and degree of aeration significantly interacted and affected the wastewater quality, while the investigated organic loading did not exert significant effect. It is suggested that combination of commercial and native inocula within wastewater would be effective, where both groups cooperate via complementary metabolic pathways, to reduce BOD and ammonia levels in domestic effluent.

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