Biological Investigation of Probiotic Industrial Waste Water Degradation for the Removal of Ammonium using Bio-Amendments

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Abstract
The flourishing global community demand for ammonium has triggered an intensification in its inventory, given that ammonium plays a pivotal role in fertilizer, pulp paper, pharmaceuticals, leather, refrigeration, photography, dyes etc. The tremendous populace usage of ammonium has led to the serious consequences for the natural nitrogen cycle, infact vandalising the environment and human health. Consequently, it is foremost to establish a technology to evaluate ammonium containing wastewaters to lower levels (<1mgNL-1) for eventual discharge to critical receiving water bodies.

Bioamendments were characterized according to the introductory concentration of ammonium present in the probiotic industrial wastewater i.e. 399.84 (~400mg/l N).

Batch experiments were conducted effectively at regular time intervals and the quantification assay of the ammonium was conducted by Kjel Dahl Unit. The results concluded that the bioamendments from a meticulous inoculum category have significantly reduced ammonium and attained 92 % accomplished higher efficiency ammonium removal rate with Chemical Oxygen Demand (COD) reduction rate as 75 % which recommends a compelling approach for the biological degradation of probiotic industrial waste water (PIWW).

Keywords: Ammonium degradation, probiotic waste water, eutrophication, waste water treatment, biological degradation, nitrogen removal.

Introduction
One of the most obligatory challenges in the 21st century is the accouterment of an adequate clean water supply that is free from pollutants or toxic waste. At the beginning of 2000, one-sixth of the global population was without access to a clean water supply, leaving over 1 billion people in Asia and Africa alone with a polluted water system.

The polluted waste water comes from major leading sources: human sewage and the process waste from manufacturing industries like all apparel, chemical and allied, electronic and electrical equipment, fabricated metal, food and kindred, furniture and fixtures, industrial and commercial machinery, leather, lumber and wood, measuring, analyzing and controlling instrument, paper and allied, petroleum refining and related, primary metal, printing, publishing and allied, rubber and miscellaneous plastic, stone, clay, glass and concrete, textile mill, tobacco, transportation equipment industries etc. which are the root causes for the promotion of eutrophication which is fatal to fish and aquatic lives and a hindrance to the disinfection of water supplies as well as having an offensive smell and carcinogenesis.

Almost every synthetic product from the above mentioned industries we use containing nitrogen atoms comes to us through the Haber-Bosch process in some way or other possible sources. All the nitrogen atoms came from ammonia. That massive carbon footprint represents a huge technological advancement, which was an energy starvation one. 1% of the world's total energy production is sucked up by the reaction which runs at 500°C temperature and up to 20 MPa pressure. According to IIP, the emitted carbondioxide levels in the year 2010 were up to 451 million tons that account for roughly 1% of global annual carbondioxide emissions, more than other industries.

Oil, natural gas or coal are used in the process reaction to produce hydrogen for ammonia synthesis for which the carbon footprint goes beyond its energy requirement. In the year 2013, combined report from IEA, ICCA and SCEB has declared that the carbon emissions by the hydrogen producing industries account for more than half of the ammonia manufacturing process.

In the global scenario, from the feedstock amassing to ammonia synthesis, every ammonia molecule manufactured will release carbondioxide as the co-product which will contribute for the carbon footprint and still our greed for the ammonia fertilizer is tremendously increasing irrespective of the energy demands.

Ammonia, the widely used chemical in most industries, is noted as one of the substantial contaminants that contributes to widespread nitrogen pollution in the hydrosphere[7].

Ammonia generally exists in the form of Non Ionised (NH_3 or Ammonia) or ionised (NH_4^+ or Ammonium) species which are the predominant pollutants in the drinking water sources. The non-ionised form is the most toxic because it is uncharged and soluble.

Mostly the anthropogenic activities are responsible for originating excess ammonia in the environment with the accelerated and uncontrolled industrialization and urbanization ,the advancements in technology and higher living standards have coupled with an augmented redundant of products leading to abundant tendency towards extravagance, which contributes to the ever increasing production of solid wastes[11].

The concentration of ammonia in the industrial wastewaters and the municipal waters may vary from 5 milligram per litre to 20,000 milligram per litre depending on its source of origin and the process undergone. They may come from the chemical fertiliser, coking, pharmaceutical, petroleum refining, coal gasification and catalyst factory[8]. On the other hand, ammonia content in the municipal wastewater is in the range of 10–200 mg/L [10].

Ammonia contributes to a noticeable upsurge of BOD in water due to oxidation process by nitrifying bacteria, which have a denoting DO requirement for the biodegradation of ammonia into nitrate. Nitrate is an end product of nitrification triggers the growth of algae and leading to the eutrophication in waterbodies[13].

The ammonium concentration quantified in water is an important indicator of the water quality index. Ammonia is rarely found in unpolluted surface water or well water, but water contaminated with sewage, animal wastes or fertilizer runoff may contain elevated levels. If the untreated polluted wastewater is discharged directly to the environment, the receiving waterbodies would become polluted and water-borne diseases accumulated with pathogenic microorganisms would be extensively scattered. The prolong exposure and direct intake of ammonia concentrations may impose adverse effects to the environment and living organisms causing various respiratory problems. For the case of acute phase survival, an individual may develop a disabling pulmonary disease.[4,5,12]

According to the WHO Reports, presence of ammonia molecule indicates the possible faeces contamination[4], ammonia threshold odour concentration in alkaline pH is ~ 1.5 mg/L and the taste concentration threshold value nevertheless is indicated as 35 milligram per liter.

According to the European Union standards, the permitted level of ammonia in the drinking water is set as 0.5Mg/l. The NAS recommends a drinking water standard of 0.5mg/l which was adopted by different nations of Europe and in the year 1990, USEP agency has declared a lifetime ammonia exposure advisory of 30mg/l. By considering the global environmental impacts of ammonia, the legislative regulations concerning the discharge of the polluted waters are drastically increasing and becoming more rigid. By considering the probable drawbacks of the ammonia release in the water streams causing tremendous ill effects, the removal of the nitrogen compounds is made mandatory. Therefore, it is not surprising that there is a growing interest in developing new technologies that are simple, cheap and highly efficient in the removal of ammonia from different waste waters.

In the past few decades, many attempts and techniques have been explored in removing high concentrations of ammonia. These processes include biological treatment, biofiltration treatment, air/steam stripping, super critical water oxidation, break-point chlorination, chemical precipitation, ion exchange andadsorption. These techniques may have advantages and disadvantages which are summarised in table 1.

There are several limitations of the current technologies, including high cost, low removal rate, high sensitivity to pH and temperature andintroducing new pollutants.

Biological treatment, the basis of wastewater treatment worldwide, was devised in the early years of 20th century and it is an accepted practice across the globe. The urge to meet stringent discharge standards has led to the implementation of a variety of advanced biological processes in the recent years.

In the present study, the monitoring and analysis of the ammonium removal are carried out using the bioamendments which will be optimized to the probiotic industrial waste water (PIWW) ammonia concentration for the feasible maximum removal rates.

Material and Methods

Probiotic waste water source and characterization: (PIWW) Probiotic industrial wastewater (corn steep liquor used as substrate for the production of powdered probiotics) was obtained from the Unique Biotech Probiotic Manufacturing Company, Jeedimetla, Hyderabad, India.

Figure 1: Showing Probiotic industrial waste water (PIWW)

Table 1
Technologies to remove ammonium from water and wastewater

Removal technologies	Removal efficiency	Advantages	Disadvantages	Ref.
Ion exchange and adsorption	80%–95%	Easy operation Effectively remove ammonium Low cost Relatively low TDS (Total dissolved solids) effluent	Certain pH ranges Different adsorbents have different removal efficiencies Require waste brine disposal	(Gupta et al., 2015; Marañón et al., 2006; Mazloomi and Jalali, 2016; Millar et al., 2016; Turan, 2016; Uğurlu and Karaoğlu, 2011; Widiastuti et al., 2011)
Biological method	70%–95%	Most commonly used method Effectively remove ammonium	High cost Require certain temperature and climate conditions High energy use High risk during subsequent processes High ammonium concentration after treatment	(Bernet et al., 2000; Feng et al., 2012; Siegrist, 1996; Thornton et al., 2007; Turan, 2016)
Air stripping	50%–90%	Widely used process for wastewater pre-treatment Simple equipment Not sensitive to toxic substances	Require certain pH, temperature, and flow rate Require large stripping towers Time consuming process High energy consuming Scaling and fouling on packings	(Guštin and Marinšek-Logar, 2011; Halling-Sørensen and Jorgensen, 1993; Huang et al., 2015a; Liao et al., 1995)
Breakpoint chlorination	80%–95%	Low spatial requirement Effectively remove ammonium Not sensitive to toxic substances and temperature Adaptable to existing facilities	Sensitive to pH Disinfection by-products High chlorine consumption under high organic matter Require highly skilled operators	(Brooks, 1999; Charrois and Hrudey, 2007; Halling-Sørensen and Jorgensen, 1993; Pressley et al., 1972; Xue et al., 2017)
Chemical precipitation	20%–30%	A valuable slow-release fertiliser Medium cost Reduce the amount of sludge and maintenance costs	Require certain pH and temperature Affect by chemical position and other ions Introduce new pollutants	(Huang et al., 2015a; Uludag-Demirer et al., 2005; Yunnen et al., 2016; Zhang et al., 2011c)
Reverse osmosis	60%–90%	Low energy requirement Low field and space Easy and continuous operation, modular construction and design simplicity	High cost Membrane elements can be fouled by colloidal matter Iron and manganese can provoke decreased scaling potential Clean the membrane regularly	(Bodalo et al., 2005; Cancino-Madariaga et al., 2011; Häyrynen et al., 2009; Noworyta et al., 2003)
Microwave radiation	~80%	Medium cost Suitable for high ammonium concentrations	Affect by pH and radiation time, initial ammonia concentration and aeration Evaporation of NH_3 Difficult for full scale application	(Lin et al., 2009; Quan et al., 2004; Rabah and Darwish, 2013)
Supercritical water oxidation	>95%	Rapid destruction of organic wastes Produce water, carbon dioxide and molecular nitrogen	High cost High temperature and pressure Affect by temperature and excess oxygen Salt precipitation	(Bermejo et al., 2008; Du et al., 2013; Kritzer and Dinjus, 2001; Marrone et al., 2004)

Table 2
Showing chemical characteristics of PIWW

S.N.	Parameters	Concentration (mg/L)
1	pH	8.5
2	Electrical conductivity	11.43 ms/cm
3	Dissolved oxygen	1.2
4	Total Organic Carbon	5976
5	Chemical oxygen demand	9200
6	Nitrates	75
7	Sulphates	83
8	Ammonium	~400 (399.8)
9	Total Solids	9800
10	Total Dissolved Solids	6400
11	Total Suspended Solids	3400

Note: *All the values are expressed as mg/l except pH

The freshly collected PIWW shown in the figure 1 which is rich in nitrogen and ammonia concentration does not require any pre-treatment and the initial chemical characteristics of the sample were carried out according to "standard methods for examination of water and wastewater 21th addition-2005, APHA [2] along with parameters shown in the table 2.

Inoculum: The Bioamendments which are prepared from the nitrification sludge, denitrification sludge, bacillus culture species, activated sludge are used as inoculum for the present study. The activated sludge was obtained from Patancheru Effluent treatment plant (PETL), Hyderabad, Telangana and nitrification sludge,denitrification sludge are collected from pre installed ammonia removal units . Raw seed sludge was filtered through a screen pore size of 2 mm to remove any fibre like material before use. Sludge was characterized to quantify total solids,total suspended solids,total dissolved solids.The inocula were stored for further degradation studies.

Experimental setup: Ammonia removal from the Probiotic industrial waste water PIWW was divided into three stages.

Introduction of Bioamendments into PIWW: Nitrification sludge (BA1), Denitrification Sludge (BA2), Bacillus Subtilis (BA3) ,Activated Sludge(BA4) are the four acclimated BioAmendments which will be introduced into four individual 1L Erlenmeyer reactor flask containing 500 ml of raw PIWW without adding any nutrients to simply the process and make it available for the readily degradation. pH is adjusted and 10% of bioamendment is added to the PIWW. Sludge volume index, sludge age and mixed liquor suspended solids are optimized.

Filtered laboratory air was provided to ensure adequate mixing and aeration. The dissolved oxygen should be maintained between 1.5-2.0 mg/l. Each series of experiments is repeated three times for reproduction of values. The ammonium concentrations are simultaneously monitored along with operational parameters and steady-state conditions.

Degradation Studies: The bioamendments (BA1, BA2, BA3, BA4) are added to the reactor flasks containing 500ml of PIWW and are placed on the shaking incubator for proper mixing and aeration. Batch studies are carried out by monitoring pH, Temperature, Ammonium Concentration, Inoculum Load, Dissolved Oxygen, Sludge Category, Total Organic Carbon, Chemical Oxygen Demand, Mixed Liquor Suspended Solids, Total Suspended Solids.

Best bioamendment for ammonia removal: Amongst all the four bioamendments,the best bioamendment which attains the maximum ammonium degradation is further isolated and used for the confirmation studies. Isolation of bioamendment is carried out by streak plate method where the procedure executes the colonies on the surface of the nutrient agar employed to bring a particular strain of microorganisms into pure culture without external culture dilution.

Analytical methods and instruments /equipment: Physical and chemical analysis were carried out for the three consecutive set of experiments. Digital pH meter for pH measurement, Conductivity meters for conductivity measurements, Hach- COD reactor for COD measurement. Ammonia quantification is estimated by using Automatic Distillation and Digestion system for kjeldhal system (Kelplus – Classic DX VATS (E), Double beam Shimadzu UV 24250 UV- visible spectro photo meter Analysis for Nitrates, Dissolved oxygen, sulphates, Total suspended solids (TSS), Total Dissolved Solids (TDS) and Total Solids conducted in accordance with American Public Health Association Standard Methods for the examination of water and Wastewater.

Results and Discussion

An exploratory trial was carried out to determine the application of Bioamendments potential for ammonium removal from probiotic industrial waste waters.

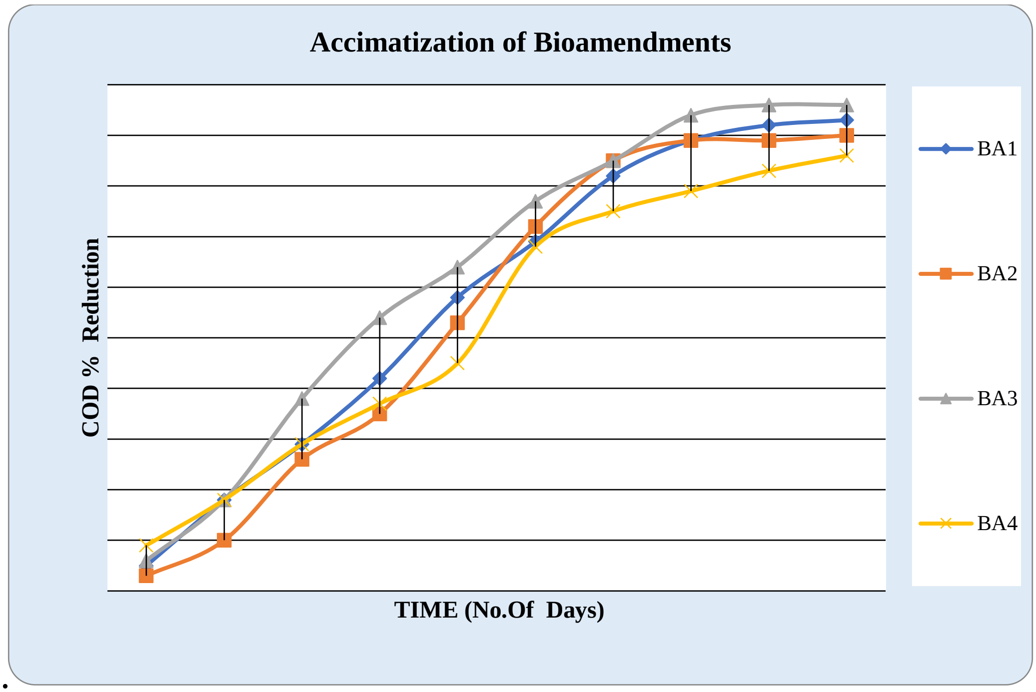
Acclimatization of Bioamendments: One of the major objectives of this study is to determine the time that bioamendments need to acclimate to ammonium .The Bioamendments - Nitrification sludge (BA1), Denitrification Sludge (BA2), *Bacillus Subtilis* (BA3) and Activated Sludge (BA4) were acclimatized with the initial concentration of 10 mg/L of NH_4-N (ammonium chloride). After acclimatization, the bioamendments were introduced into the reactor flasks for the further degradation studies. Graph 1 shows that the bioamendments (BA1, BA2, BA3, BA4) are showing a great COD % reduction which indicates the maximum acclimized state of the four amendments.

Effect of Dissolved oxygen: The concentration of dissolved oxygen in the reactor flask was maintained approximately 1.1 mg/L (Table 2). Theoretically with this value, ammonia removal may be carried out without difficulty since the minimum value of dissolved oxygen must be 0.5 mg/L. However, efficiency is much better when dissolved oxygen levels are maintained between 0.5-2.0 mg/l as this guarantees that the oxygen penetrates the interior of the flocs formed by the bacteria.

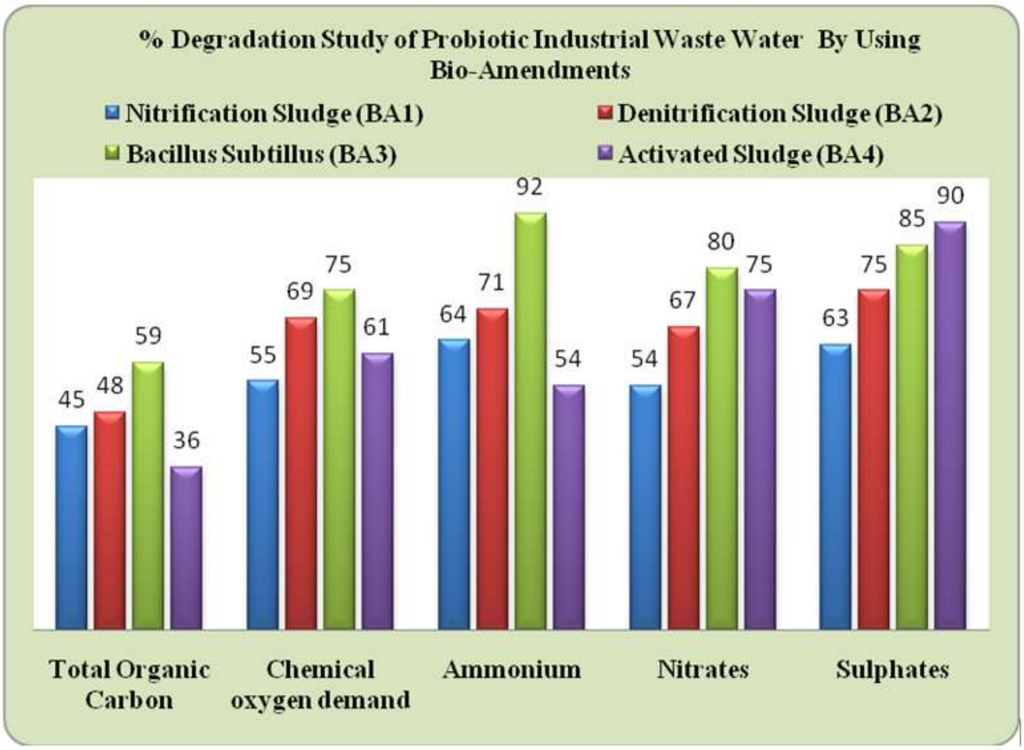
Effect of temperature: The temperature is an important factor of microbial survival, it influences the growth of microorganism and the absorption and utilization of growth substances[6] mainly through changing the activity of the enzyme. It can be seen that the optimal values of temperature were observed as 35˚C.

Effect of pH: The pH values of the culture media affect microbial development. Having pH values that are either too high or too low are not conducive to cell growth[9,14]. When the pH value ranged from the 7.5 to 8.0, the degradation rate kept increasing. The degradation rate reached a maximum of 92% at pH 7.8. After that, the degradation rate then decreased. This indicated that partial alkaline environment was conducive to the degradation of ammonium. It was proven that optimal ammonia degradation rates occur at pH values in the 7.5 to 8.0 range, therefore a pH of 7.0 to 7.2 is normally used to maintain reasonably nitrification rates.

Degradation studies: Three consecutive set of experiments were conducted as Function of pH, Contact time, Ammonium Concentration, Inoculum Load, Dissolved Oxygen, Sludge Category, Total Organic Carbon ,Chemical Oxygen Demand, Mixed Liquor Suspended Solids, Total Suspended Solids for the biodegradation of Probiotic industrial waste water (PIWW) to systematically evaluate the treatability of the degradation process by using bioamendments.



Graph 1: Showing Acclimatization of Bioamendments



Graph 2: Showing Degradation study of PIWW by using Bioamendments

Figure 2: Isolated colonies of Streaked Bioamendment (BA3)

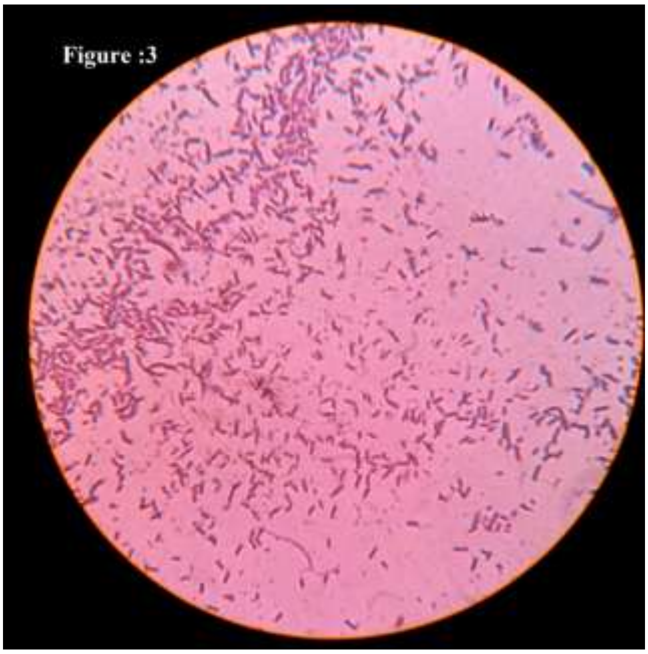


Figure 3:Microscopic Observation of Bacillus Subtillus (BA3) Bioamendment

Table 2
Ammonia Degradation % Removal studies by using Different bioamendments at 10 hrs HRT

			Name of Bioamendments (% Removal Efficiency)			
S.N.	**parameters**	**Initial Concentration**	**Nitrification sludge (BA1)**	**Denitrification sludge (BA2)**	**Bacillus subtillus (BA3)**	**Activated sludge (BA4)**
1	Total Organic Carbon	5976 mg/l	45	48	59	36
2	Chemical oxygen demand	9200mg/l	55	69	75	61
3	Nitrates	75 mg/l	54	67	80	75
4	Sulphates	83 mg/l	63	75	85	90
5	Ammonium	~400 (399.8) mg/l	64	71	92	54
	pH	pH was maintained between the 7.5 to 8.0 range				
	Dissolved oxygen	Dissolved oxygen was maintained between 0.5-2.0 mg/l				
	Temperature	Temperature was maintained at 35^o C				

The chemical oxygen demad (COD) maximum removal efficiency of 75% (2300 mg/l COD) was obtained with BA3 (*Bacillis subtillus*) at 10hrs HRT and least % removal was observed at 55% (4100mg/l COD) at 10hrs HRT with BA1 nitrification sludge. Results indicate that more COD removal can be achieved at longer rention time and with higher initial COD loading concentration of 9200mg/l. This condition might be due to bioamendments which get higher contact time to convert organic substrate to biomass to produce more removal efficiencies.

The maximum ammonium (92%) and COD (75%) removal efficiencies was reported by BA3 *Bacillus subtillus* which can be used as the best BioAmendment for the ammonium removal concentrations ranging to 400mg/l.

Isolation of the Best Bioamendment to confirm the Maximum degradation of ammonium: By using aseptic technique, the selective and best bioamendment (BA3) *Bacillus subtillus* from the reactor flask was streaked on the agar Petri plate. The Petri plate divided into 4 parts is streaked and sealed and which is kept in 30°C incubator for growing. After 24 hours, the isolated colonies of *Bacillus subtillus* appear on the Petri plate as in figure 2.

Confirmation of Bioamended microorganism: For the confirmation or identification studies, pick an isolated colony from the Petri plate and perform biochemical identification methods namely gram staining, a standard procedure based on Bergey's manual which is constitutional to the phenotypic characterization of bacteria , differentiates organisms of the domain Bacteria according to the cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple as in figure 3. Gram-negative cells have a thin peptidoglycan layer and stain red to pink. After gram staining test, the microscopic observation confirms the rod shaped, gram positive *Bacillus subtillus*, a Bioamendment used in this study from the figure 3 which has removed maximum ammonium from the probiotic industrial wastewater (PIWW)

Conclusion

The consecutive set of batch experiments with acclimized sludge and optimized parameters performed at 10hrs HRT by using bioamendments proved to be rewarding with excellent ammonium quantification concentration of 92 % with a COD removal efficiency of 75% by BA3 Bioamendment. Based on the result obtained from the study of isolation and identification of the bacteria, *Bacillus subtillus* was identified for further reconfirmation stating as best bioamendment which can be applied efficiently to treat the probiotic ammonium waste water ranging ~400mg/l

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