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CONTENTS

Research

Crop Effects on Lead Fractionation in a Soil Treated with Lead Organic and Inorganic Sources

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ABSTRACT: The purpose of this study was to determine lead (Pb) fractionation in a sandy loam soil (Typic Xerorthents) amended with enriched (600 mg Pb kg^{-1}) biosolids and cow manure before and after cultivation of sunflower (*Helianthus annuus* L.) and corn (*Zea mays* L. Single grass 704). In the salt treatment the Pb bounded to oxide fraction was higher (8.7%). Pb bounded to the carbonate fraction was lower (12%) in the sunflower rhizosphere than in the corn rhizosphere. Corn cultivation in the biosolids treatment displayed a 10% increase in the residual fraction with a 6% decrease in oxide fraction. Crop cultivation had no significant effect on ratio of exchangeable to carbonate fraction in biosolids-amended soil. However, ratios of carbonate to oxide and oxide to residual fractions decreased in the sunflower rhizosphere soil as compared to the uncultivated soil.

Cultivation of sunflower in the manure-amended soil had no significant effect on the carbonate to oxide and oxide to residual fractions' ratios. Corn plants grown in soil treated with manure accumulated lower Pb in their roots versus those grown in the biosolids-treated soil. In soils treated with biosolids and inorganic Pb salt, corn accumulated greater amounts of Pb in its roots compared to the sunflower, whereas both crops had similar root Pb concentration when they grew in manure amended soil. A high correlation (> 92%) was observed between Pb uptake by crops grown in soils treated with biosolids and cow manure and metal concentration in soil inorganic fractions. Phototoxic effects of Pb were clearly observed on the sunflower grown in Pb salt treated soil while no such effect was found for plants grown in soils treated with sludge and manure.

1. INTRODUCTION

LAND application of organic amendments such as biosolids (i.e., sewage sludge) and cow manure is both environmentally and economically advisable [32]. Application of organic amendments enables recycling of valuable components such as organic matter (OM) and plant nutrients [18, 19]. On the other hand, these amendments may contain high levels of toxic metals, which limit their land application due to possibility of food chain contamination. High loading of organic amendments to soil may give rise to an accumulation of heavy metals. Increase of heavy metals concentration in soil could lead to phytoxicity for plants, contamination of food chains and ground and surface water resources [34, 36].

Heavy Metal bioavailability in soils from organic amendments varies widely and is affected by many of soil parameters such as pH and type of soil minerals. However, total metal concentration provides little indication of metal bioavailability, mobility, and reactivity in soils received organic amendments [4, 20]. Heavy metals are non-biodegradable and occur in various forms in organic amendments. Metals mobility and bioavailability in a given medium has to be determined to assess environmental impact.

Metals in soils, sediments, and wastes occur in several different physico-chemical forms (e.g., simple or complex ions, easily exchangeable ions, organically bound, occluded or coprecipitated with metal oxides, carbonates, phosphates, secondary minerals, or ions in crystal lattices of primary minerals) [15]. Many different sequential extraction procedures have been applied to evaluate contamination risk for soil [33, 42] and sediment [22, 29]. In all sequential extraction schemes extractants are applied in order of increasing reactivity. Therefore, successive fractions obtained correspond to metal association forms with lesser mobility and bioavailability. An extended method was developed by

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Tessier et al. (1979) [42]. Tessier et al. (1979) performed fractionation of metals in samples of sediments into 5 parts: (1) exchangeable fraction representing most easily available metals, (2) carbonate fraction, (3) Fe, Mn, and Al oxides fraction, (4) organic matter fraction, and (5) residual fraction, tightly bound on silicate matrices of samples. Exchangeable and carbonate metal forms are considered readily mobile and available to plants, while metals incorporated in clay crystalline lattices seem to be relatively inert. Other metal forms, associated to Fe, Al, and Mn oxides or those bound with organic matter could be considered potentially active or strongly bound, depending on soil physical properties [3, 11, 12, 13]. Small changes in experimental conditions (e.g., pH, temperature, contact time, solid to extractant volume ratio, particle size and sample pretreatment) can lead to large variations in fractionation, making it troublesome for comparisons between results. On the other hand, crop cultivation may change distribution of metals. Relationships between the chemical fractionations of trace elements in soils and plant uptake have been estimated by simple and multiple correlation procedures. For example, simple correlations between chemical fractionations and plant metal contents were observed. Exchangeable [21, 44] and organically bound [5] trace elements were found to predict plant tissue concentrations reasonably well.

Crop cultivation may change metal availability via different mechanisms such as exudation of complexing agents, roots respiration and reduced soil pH [8]. Although effect of plant cultivation on soil fractionations for some trace metals has been investigated, limited information is available on soil Pb fractionations as affected by crop cultivation or organic amendments [16]. Therefore, this study was conducted to determine Pb fractionation in a soil amended with Pb-enriched biosolids, cow manure and $Pb(NO_3)_2$ salt in relation with Pb concentration in corn and sunflower tissues.

2. MATERIAL AND METHODS

2.1. Experimental Design and Rising of Plants

A loamy sand, non-saline, non calcareous soil (Typic Xerorthents) [40] with low organic carbon content was collected from soil surface layer (0–15 cm) around Shahr-e-kord, central Iran. Soil was air-dried and ground to pass a 2 mm sieve. Selected properties of soils are presented in Table 1.

Table 1. Selected Physico-chemical Properties of Unamended, Biosolids and Cow Manure Amended Soil.

Parameter	Unit	Unamended Soil	Biosolids Amended Soil*	Cow Manure Amended Soil*
pH	—	7.3	6.8	7.3
EC	dS m^{-1}	2	3.3	5
Organic carbon	%	0.1	2	2.7
Sand	%	70	—	—
Silt	%	18	—	—
Clay	%	12	—	—
Iron oxide	mg kg^{-1}	600	620	600
Total P	%	0.01	0.03	0.02
$CaCO_3$	%	10	10	11
Total Pb	mg kg^{-1}	3	580	570
CEC	cmolc kg^{-1}	31.3	33	34.7

*Enriched with $Pb(NO_3)_2$ to about 600 mg Pb kg^{-1}

Biosolids used in this experiment were a secondary, anaerobically digested municipal biosolids from the city of Isfahan, central Iran. Cow manure was 8-months decomposed. Biosolids and manures were enriched with $Pb(NO_3)_2$ to a 600 mg Pb kg^{-1} level and incubated for two weeks. Basic properties of organic amendments are displayed in Table 2.

A similar rate of Pb (600 Pb kg^{-1}) was added to soil through two organic amendments (i.e., enriched biosolids and manure) and an inorganic salt ($Pb(NO_3)_2$). Enriched biosolids and manure were applied at a rate of 10% by weight to the soil. Four kilograms of treated soils were put into plastic pots. Soil moisture was kept at 80% water holding capacity during incubation at 23–25°C for two weeks. Sunflower (*Helianthus annuus* L.) and corn (*Zea mays* L. single grass 704) seeds were sown with 3 seedlings kept in each pot.

2.2. Samples Collection and Analyses

After 60 days, plants were harvested by cutting shoots at the soil surface and removing roots from pots.

Table 2. Selected Chemical Properties of Biosolids and Cow Manure.

Parameter	Unit	Biosolids	Cow Manure
EC	dS m^{-1}	9	17
pH	—	6.7	8.9
Organic carbon	%	17.9	31.3
Fe_2O_3	mg kg^{-1}	730	73
Total Pb	mg kg^{-1}	75	20
Total Zn	mg kg^{-1}	710	217
Total Cd	mg kg^{-1}	5	3

Shoots and roots were washed with tap water, rinsed with deionized water, and dried at 65°C for 24 h. Thereafter, plant samples were combusted over night at 480°C. Ash was treated with HNO_3 and heated to near dryness, and the sample dissolved in 3 M HC1 with heating. Lead concentration in extracted solutions was measured using atomic absorption spectrophotometry (AAS) (Perkin Elmer model 3030).

Total Pb in soil and organic amendments was extracted using a tri-acid mixture (HNO_3:H_2SO_4:$HClO_4$ 5:1:1) and then determined on atomic absorption spectrometry (AAS) [2]. Soil pH was measured using a digital pH meter (Model 691, Metrohm AG Herisau Switzerland) [43] and electrical conductivity (EC) was measured with an EC meter (Model Ohm-644, Metrohm AG Herisau Switzerland) [31]. The $CaCO_3$ equivalent was determined by neutralizing with HCl and back titration with NaOH [25]. Organic matter content was determined by the Walkley and Black method [24]. Soil texture was determined according to a method presented by Gee and Bauder (1986) [7]. Total soil P was determined by a colorimetric method [26]. Cation exchange capacity (CEC) was measured using the Rhoades (1982) method [30].

2.3. Translocation Factor

Ability of plants to transport heavy metals from roots to shoots was measured by calculating translocation factor (*TF*) as displayed in Equation (1) [39]:

$$TF = H_r / H_s \quad (1)$$

where H_r and H_s are heavy metal concentrations in shoot and roots, respectively.

2.4. Sequential Extraction

Five-step sequential extraction scheme developed by Tessier et al. (1979) (Table 3) was used to determine Pb distribution in different fractions [42]. An internal check was performed on samples for comparing total metal removed by sequential extraction with total digestion results according to Tessier et al. (1979) [42]. Sample recovery was calculated using the following formula [Equation (2)]:

$$\text{Recovery} = [\text{soluble} + \text{exchangeable} + \text{carbonate} + \text{oxide} + \text{organic} + \text{residual}]/\text{total Pb} \quad (2)$$

Pb recovery in the five steps sequential extraction procedure ranged between 90 to 95% of total metal concentration.

Table 3. Extraction Conditions Used per Gram of Soil According to the Fractionation Scheme of Tessier et al. (1979) [42].

Exchangeable	8 mL 1M $MgCl_2$ (pH=7), 1 h, room temperature, continuous agitation
Carbonate	8 mL 1 M NaOAc (pH=5), 5 h, room temperature, continuous agitation
Oxide	20 mL 0.04 M $NH_2OH{\cdot}HCl$ in 25% HOAc, 5 h, 96°C, some agitation
Organic	3 mL 0.02 M HNO_3 and 2 mL 30% H_2O_2 (pH= 2), 2 h, 85°C, some agitation; additional 3 mL 30% H_2O_2, 3 h, 85°C, some agitation; 5 mL 3.2 M NH_4OAc in 20% HNO_3 , 0.5 h, room temperature, continuous agitation
Residual	0.5 mL conc. HNO_3 , 5 mL HF and 2 mL HCl, digestion in Teflon bomb, dissolution in 15% HCl
Total	Triple acid attack of 0.5 mL conc. HNO_3 + 5 mL HF +2 mL HCl, digestion in Teflon bomb, dissolution in 15% HCl

Accuracy of Pb analysis was controlled by following certified standards from the National Institute of Standards and Technology (NIST) and by including blanks in digestion batches.

2.5. Mobility Factor

Soil's Pb mobility factor may be assessed on the basis of absolute and relative content of fractions weakly bound to soil components. The relative index of metal mobility was calculated as a mobility factor (MF) [23] on the basis of the Equation (3).

$$MF = (F1+F2+F3)/(F1+F2+F3+F4+F5+F6) \quad (3)$$

where F1, F2, F3, F4, F5, and F6 are water soluble, exchangeable, carbonate, oxide, organic, and residual fractions (mg Pb kg^{-1}) in soil, respectively.

2.6. Statistics

A factorial experiment using completely randomized design was used in three replications. Statistical analyses were performed using the ANOVA procedure [35]. Differences between means were evaluated using the least significant difference (LSD). The 0.05 probability value was used to determine significant difference. In addition, a step-wise multiple regression analysis between sunflower tissue Pb concentration and corn and Pb concentration in soil fractions was performed by using SAS software [35].

3. RESULT AND DISCUSSION

3.1. Organic Amendments Effects on Soil Properties

Soil application of biosolids and cow manure increased the CEC of the rhizosphere soil by 1.7 and 3.4 units and also increased soil organic C from 0.1% to 2% and 2.7%, respectively (Table 1).

Adding cow manure to soil had no effect on soil pH, while biosolids application resulted in a 0.5 unit decrease in soil pH. The small change in soil pH is probably due to a high buffering capacity of the soil and relatively high calcium carbonate content (Table 1).

The electrical conductivity (EC) of cow manure was much greater than that of biosolids, thus, soil treated with manure had higher EC versus soil treated with biosolids and $Pb(NO_3)_2$ salt.

3.2. Mobility Factor (MF)

Regardless of crop type, the Pb MF in the salt treatment was significantly greater than from organic amendments' treatments (Table 4). Accordingly, sunflower plants grown in $Pb(NO_3)_2$-treated soil displayed leaf necrosis symptoms induced by Pb toxicity while no such symptoms were observed on plants grown in biosolids and manure treatments. This result demonstrates that organic fractions of organic amendments play an important role in increasing soil adsorptive capacity and thus, reducing metal mobility and phytoavailablity [9, 10].

Regarding inorganic Pb treatment, corn cultivation significantly increased Pb MF compared to the uncultivated initial soil, while cultivation of sunflower had no significant effect on Pb MF (Table 4). Significant increase of Pb MF in the corn rhizosphere soil is probably due to rhizospheric environment that may partially change soil properties such as soil pH. Data are not displayed. However, greater availability of organic ligands, especially low molecular weight exudates or some other unique chemical characteristics of rhizosphere may also be considered [1, 17].

3.3. Root and Shoot Pb Concentrations

Plant Pb concentration was largely affected by both organic treatments applied and plant species (Table 5). In Pb salt treatment, shoot and root Pb concentration in corn was greater than in sunflower, but Pb toxicity effects (i.e., reduction in plant height and dry biomass, and appearance of leaf necrosis spots) were more severe in sunflower. Pb toxicity symptoms were similar with symptoms reported by Tandy et al. (2006), Kosobrukhov et al. (2004) and Chantachon et al. (2004) [41, 14, 6] . Inhibition of shoot growth by Pb toxicity may be due to a decrease in photosynthesis, imbalanced mineral nutrition, impaired water uptake, changes in hormonal status, and increased membrane permeability [37].

Corn plants grown in manure treatment accumulated lower Pb in root than those grown in biosolids treatment (Table 5). This is most likely attributed to higher affinity of manure to adsorb Pb compared to biosolids because of greater organic carbon content in manure. In addition, cow manure had a higher pH (Table 2) that may cause precipitation of Pb and thus decreasing its phytoavailability.

Lead concentrations in sunflower and corn root cultivated in biosolids and manure treatments were significantly lower than those grown in soil that received inorganic Pb salt (Table 5). Regardless of crop species, application of manure decreased root Pb concentration by nearly 70% in comparison to the inorganic Pb salt treated soil. Metal accumulation in roots may be due to a specific strategy used by a plant for storing and inactivating accumulated toxic elements in root cell walls [27] or it can be ascribed to complexation of heavy met-

Table 4. The Mobility Factor (MF) of Pb in Soil as Affected by Crop Cultivation and Application of Organic and Inorganic Pb Sources.

Treatment	Before Cultivation*	After Cultivation	
		Corn	Sunflower
$Pb(NO_3)_2$-received soil	0.57^{b}	0.69^{a}	0.56^{b}
Cow manure amended soil	0.22^{c}	0.17^{c}	0.23^{c}
Biosolids amended soil	0.17^{c}	0.19^{c}	0.10^{d}

*Means followed by the same letter are not significantly different ($p = 0.05$).

Table 5. Shoot and Root Pb Concentrations of Sunflower and Corn Grown in Soils Treated with $Pb(NO_3)_2$, Cow Manure, and Biosolids.

Treatment	Corn**		Sunflower	
	Shoot	Root	Shoot	Root
$Pb(NO_3)_2$-received soil	25.0^{f}	335^{a}	23.2^{g}	323.2^{b}
Cow manure amended soil	7.7^{i}	91.5^{d}	12.25^{h}	92.0^{d}
Biosolids amended soil	nd*	99.2^{c}	22.2^{g}	31.5^{e}

*nd: not detectable

**Means followed by the same letter are not significantly different ($p = 0.05$).

als with sulphydryl groups resulting in less translocation of metals to shoots [38]. Li et al. (2007) indicated Pb concentration was much higher in roots than in straw or grain with the lowest Pb concentrations being found in rice grain [16].

3.4. Pb Translocation Factor (TF)

The Pb concentration in the aerial part of corn plants grown in biosolids treated soil was less than the AAS detection limit. Therefore, the TF value was not calculated for corn in this treatment.

Regardless of Pb source, the TF value of Pb was frequently less than 1 for both corn and sunflower (Table 6). Except in inorganic Pb salt treatment, the TF value of Pb was higher for sunflower than for corn. The highest TF value was found in sunflower plants grown in sludge-treated soil. No relationship was found between metal toxicity symptoms and TF values. Therefore, no Pb toxicity injuries were found on plants grown in soils treated with manure and sludge whereas in Pb salt treatment, despite the lowest TF values, symptoms of Pb toxicity were observed on sunflower plants.

Table 6. The Pb Transfer Factor (TF) of Sunflower and Corn Grown in Soils Treated with $Pb(NO_3)_2$, Cow Manure and Biosolids.

	TF	
Treatment	**Corn****	**Sunflower**
$Pb(NO_3)_2$-received soil	0.07	0.07
Cow manure amended soil	0.08	0.13
Biosolids amended soil	nd*	0.70

*nd: not detectable
**Means followed by the same letter are not significantly different ($p = 0.05$).

3.5. Lead Fractionation in Soil

Adding cow manure and biosolids to a soil resulted in a significant increase in oxide and residual Pb fractions, while carbonate fraction decreased by 34% and 41%, respectively (Figure 1). Manure treatment had higher amounts of Pb (i.e., percentage) bounded to carbonate fraction versus that from biosolids' treatments. Cow manure and biosolids' application increased the ratio of exchangeable to carbonate, but the ratio of carbonate to oxide fraction decreased. Contribution of more than 55% inorganic (oxide) fraction in biosolids and manure-treated soils indicates an important role of inorganic fraction in Pb adsorption capacity.

Lead distribution in different fractions under corn and sunflower are displayed in Figure 1. In soil treated with $Pb(NO_3)_2$ salt, the Pb concentration in organic fraction was below ASS detection limits (Figure 1). Nearly 63% and 27% of total Pb concentration in soil under corn was extracted in carbonate and oxide fractions, while for sunflower-cultivated soil, about 50%

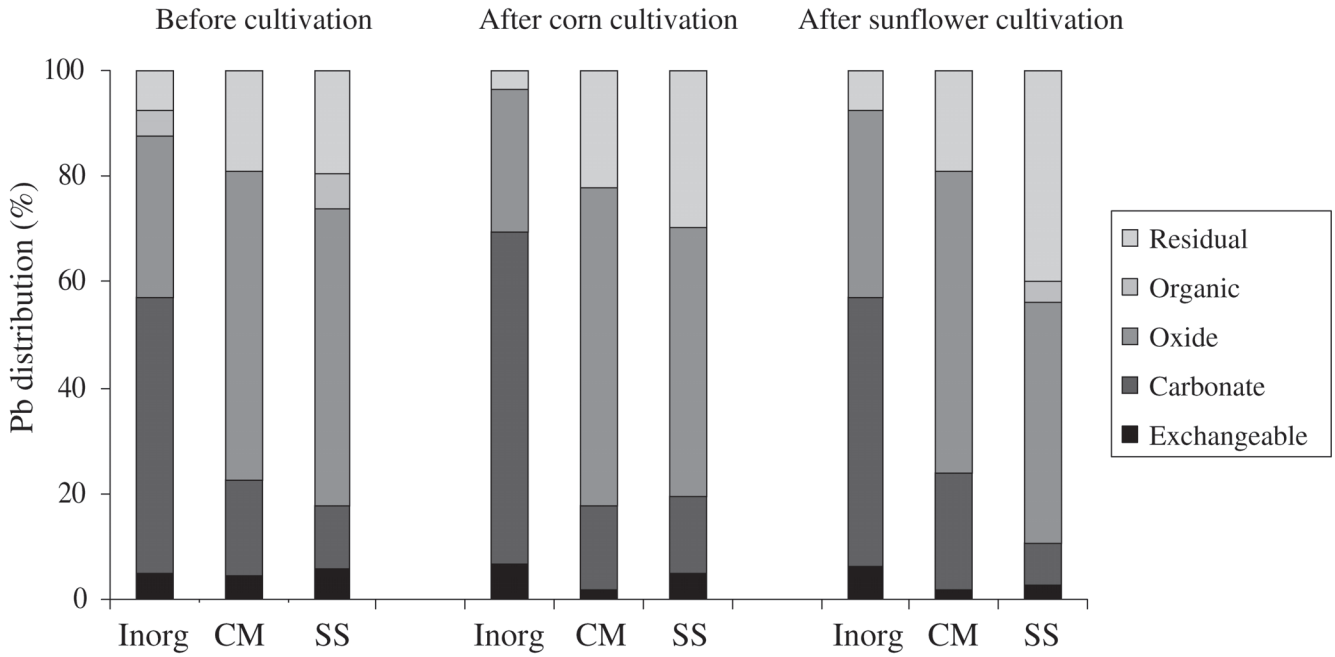

Figure 1. *The Pb fractionation in soil amended with biosolids (SS), cow manure (CM) and $Pb(NO_3)_2$ salt (Inorg) before and after cultivation of corn and sunflower.*

and 35% of total Pb was found in carbonate and oxide fractions, respectively. Cultivation of corn in $Pb(NO_3)_2$-treated soil increased the carbonate fraction by 10%, whereas it caused a 4% decrease in oxide and residual fractions. Accordingly, the MF of Pb increased in the corn rhizosphere as compared to uncultivated soil (Table 4). Corn cultivation in soil receiving Pb salt resulted in an increase in the ratio of exchangeable to oxide and carbonate to oxide fractions, whereas it had no significant effect on the exchangeable to carbonate ratio. Lin et al. (2004) reported that NH_4OAC Pb extractable in the rice rhizosphere was significantly greater than bulk soil [17]. On the other hand, Pérez-de-Mora (2007) reported that plant cover had no effect on Pb distribution in soil [28].

Cultivation of sunflower in soil treated with $Pb(NO_3)_2$ increased the Pb content of the oxide fraction by 8.7%, while it decreased Pb carbonate fraction by 12%. In $Pb(NO_3)_2$-treated soil, crop type had no significant effect on the ratio of exchangeable to carbonate fractions. However, the ratio of carbonate to oxide fraction of Pb in sunflower rhizosphere was lower than corn rhizosphere.

In biosolids treatment, Pb fractionation was significantly affected by crop species (Figure 1). Corn cultivation in soils treated with biosolids caused a 10% increase in residual fraction and 6% decrease in oxide fraction as compared with uncultivated soil. On the other hand, sunflower increased residual Pb fraction by 11% and decreased oxide fraction of Pb by 20%. Greater decrease in the residual fraction of soil Pb by sunflower compared to corn may be partly explained by lower root Pb concentration for sunflower versus corn in biosolids treatment. Although, the high transfer factor for sunflower resulted in greater transport of Pb from root to shoot and, thus, higher shoot content for sunflower versus corn.

Although shoot Pb concentration of sunflower plants grown in the $Pb(NO_3)_2$ treated soil was similar to those grown in the soil treated with biosolids, toxicity symptoms were more clearly observed on sunflower plants in the inorganic Pb salt treatment versus in the biosolids treatment. This result suggests Pb added to soil by inorganic $Pb(NO_3)_2$ salt is more bio-available in this case than metal added to soil by biosolids.

Crop cultivation had no significant effect on ratio of exchangeable to carbonate fraction in biosolids amendment soil, although ratios of carbonate to oxide or oxide to residual fractions of Pb in the sunflower rhizosphere was lower than in the corn rhizosphere. Cultivation of corn in biosolids treatment had no effect on the order distribution for oxide, residual, and carbonate fractions, but significantly decreased the organic Pb fraction (Figure 1).

Cultivation of corn in cow manure amended soil caused a significant increase in Pb concentration of residual fraction, but decreased the exchangeable fraction by 3% compared with the uncultivated soil (Figure 1). This decrease in the exchangeable fraction of soil Pb may explain the significant decrease in root Pb accumulation of corn plants grown in manure-treated soil compared with those grown in soil treated with biosolids (Table 6). In contrast to root, shoot Pb concentration in corn plants grown in manure was greater than those grown in biosolids. This is partly due to greater shoot dry matter yield of corn plants in the biosolids treatments and, thus, diluted metal concentration (dilution effect) (data are not shown).

Regardless of crop type, plant cultivation in soil treated with cow manure caused approximately a 3-fold decrease in the ratio of exchangeable to carbonate fraction (Figure 1). It has been shown that metal concentration in soil exchangeable fraction can give an estimate of metal phytoavailability [21, 44]. Therefore, reduced Pb content of exchangeable fraction is a reason for lower Pb uptake by studied crops in the manure-treated soil versus soils treated with biosolids and particularly inorganic Pb salt.

A linear correlation suggested root Pb uptake by sunflower or corn was positively related to carbonate and oxide fractions (Table 7). Running a step-wise multiple regression analysis demonstrated that 99% variation of root Pb uptake by sunflower or corn was explained by carbonate fraction followed by oxide fraction. This result indicates the importance or role of the inorganic fraction in Pb phytoavailability. For sunflower, the shoot Pb concentration was significantly correlated (r^2 = 0.92) with exchangeable and residual fractions of Pb, whereas for corn, the shoot Pb concentration was correlated (r^2 = 0.99) with Pb carbonate fraction. This is in agreement with findings from Kabala and Singh

Table 7. Equations and Correlations Coefficients Between the Tissue Pb Concentration of Sunflower and Corn and Pb Concentration in Soil Fractions.

Plant	Equation	R^2
Sunflower	Pb_{root}=1.65 car-Pb- 0.84 Ox-Pb + 150.90	0.99
	Pb_{shoot}=0.42 Exch-Pb +0.075 Res-Pb	0.92
Corn	Pbroot= 1.31car-P- 0.42Ox-Pb +110.440.99	0.99
	Pb_{shoot}=0.147 Car-Pb 0.95	0.95

(2001), suggesting carbonate and exchangeable metal fractions are readily bioavailable pools to plants [13].

4. CONCLUSION

Fractionation of Pb in soil is a useful technique for determining effect of organic amendments on metal phytoavailability in plants and is also useful for investigating the relative importance of organic and inorganic phases of amendments regarding retention/sorption of toxic metals. Organic amendment application had less effect on increasing Pb availability compared to inorganic amendment application. Adding Pb salt to the soil significantly increased the carbonate fraction that is bioavailable for plants, whereas in soil treated with organic amendments, most of the total Pb was in the oxide fraction. Plants grown in soils treated with inorganic Pb accumulated greater Pb concentration in their root and shoot compared to those grown in biosolids and manure-amended soils. Crop cultivation significantly affected Pb fractionation. Results indicated that in organic-amended soils more than 45% of Pb was in the oxide fraction. Despite the greater organic carbon found in cow manure versus that found in biosolids, the percentage of inorganic fraction in manure-treated soil was higher than in soil treated with biosolids. The Pb toxicity symptoms were more evident on sunflower plants grown in inorganic Pb salt-treated soil versus those grown in soils treated with biosolids and manure. This indicated that Pb accumulation in plant tissues largely depends on the type of organic treatment applied and plant species. According to results, it seems risk of Pb transfer to a food chain in soils treated with organic amendments is much less than it is for soils with Pb received via inorganic sources.

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Use of EDTA and EDDS for Enhanced Zea Mays' Phytoextraction of Heavy Metals from a Contaminated Soil

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ABSTRACT: Application of EDTA and EDDS (5 and 10 mmol kg^{-1}) had inhibitory effects on growth of zea mays. Treatments of ED-5, ED-10, ET-5, and ET-10 for Cd resulted in 16.1, 17.5, 22.5, and 24% reduction and for Pb 1.7, 14.3, 6, and 18.3% reduction in shoot dry biomass, respectively, compared to the control. Concentrations of Cd in shoot were increased 2.9, 3, 2.4, and 2.5 times and for Pb 1.6, 1.7, 3.4 and 7.9 times, respectively. Sequential extraction indicated application of these two chelates increased exchangeable Cd (1, 1.1, 1.6, and 1.8 times) and Pb (2.2, 3.2, 5.8, and 12.8 times). Generally, the most effective chelators for Cd accumulation and phytoextraction was 10 mmol kg^{-1} EDDS. As for Pb, the addition of 10 mmol kg^{-1} EDTA was used to obtain maximum amount of phytoextraction.

1. INTRODUCTION

DUE in large part to rapid advancement of agriculture and industry in many parts of the world, soil contamination by heavy metals has become a reoccurring major environmental issue. Elevated concentrations of heavy metals not only lead to reductions in soil microbial activity, soil fertility, and in crop production [17], but also threaten human health and interests through the food chain [16]. Elevated concentrations of metallic elements in various soils are primarily a result of anthropogenic activities, such as mining and smelting industries, sewage sludge application, employment of mineral fertilizers, and to some extent consumption of leaded petrol in the past [1].

Cadmium (Cd) is a common heavy metal pollutant in mine lands [39], which is of great concern in the environment due to its toxicity. Concentrations of Cd can accumulate in plants causing no harm to the plant and yet they are toxic to animals eating the contaminated plant. Cadmium toxicity, particularly, affects human beings rather than animals. This is attributed to their longer life span and gathering of Cd in their organs by eating Cd-contaminated food [34]. Lead, a common pollutant, is of concern because it is not a plant nutrient, but is potentially toxic to animals and humans [13]. It ranks second among the most hazardous metals on the US Environmental Protection Agency (EPA) Priority List [16].

Traditional remediation methods of such contaminated soils (e.g. excavation and discarding of soil) have generally proven costly and harmful to soil properties [22]. Phytoextraction using engineered metal-accumulating plants for removal of toxic metals from soil is an environment-friendly and cost-effective technology compared to common conventional remediation techniques [11].

In soil conditionally and depending on chemical properties of pollutants, soil properties, environmental conditions, and biological activities [28], it is common to find cases of low metal bioavailability (e.g. Pb). This is an obstacle for the process of phytoextraction [2].

For successful phytoextraction of metals from the soil two different strategies have been proposed. The first uses hyper/ accumulator plants that can naturally extract large amounts of metals from the soil [18]. The second employs high biomass, non-hyperaccumulator plants which are induced to absorb large amounts of metals from soils that have had their metal solubility increased by application of chemical mobilizing agents such as citric acid, ethylenediaminetetraacetic acid

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(EDTA), and nitrilotriacetic acid (NTA) [8]. Some agricultural crops (e.g., Brassica napus L., Brassica juncea L., Cannabis sativa L., Helianthus annuus L., Phaseolus vulgaris L., Sinapis alba L., and Zea mays L.) were found to be effective in removing metals [3,9].

Among chelator agents, EDTA has particularly been the focus of attention as a soil amendment in phytoextraction researches. It is a complex agent used in agriculture as an additive in micronutrient fertilizers since the 1950s [20]. Even though this chelate increases metal mobility and availability of the latter, the amount taken into solution is higher than the capability of the plant to amass [16]. On the other hand, EDTA can maintain itself in the soil for several weeks, and EDTA-mobilized metals may result in a leaching down of the soil profile over this period of time [13], Thus, the spotlight in recent times has been on the use of chelating agents such as ethylenediaminedisuccinate (EDDS) and others. Once they have accomplished the intended purpose of transporting metals in the soil in a controlled manner, they readily biodegrade [5, 32]. EDDS, a structural isomer of EDTA, owns two chiral carbon atoms and three stereo isomers [35], Among them, only the (S,S) isomer is readily biodegradable. EDDS has the potential of being EDTA's substitute in chelant-assisted phytoextraction due to the fact it is a strong chelator and unlike EDTA, is easily biodegradable [27]. Several recent papers have been devoted to investigating of use of EDDS in chelant-enhanced phytoextraction of metals [15, 19].

The aim of this study is to compare the efficiency of two chelators EDTA and EDDS for the enhancement of metal uptake and translocation by "Zea mays" to remediate heavy metals in polluted soils.

2. MATERIALS AND METHODS

2.1. Greenhouse Experiment

Surface samples (i.e., 0–30 cm) of air-dried, homogenized, and sieved (10 mm) soil (7 kg) were artificially amended with Pb and Cd, then fertilized with 100 mg kg^{-1} N ($(NH_4)_2SO_4$), 100 mg kg^{-1} K (K_2SO_4), and transferred to plastic pots 27 cm in height and 25 cm in diameter. Three seeds of Zea mays were planted into each pot. Three replicates were used for each treatment. EDTA and EDDS were applied in different doses 9 weeks after seed germination. Treatments included a control (i.e., soil with no chelator) and 2 chelators amended to the soil's surface in solutions at doses of 5 and 10 mmol kg^{-1} dry weight (DW). Soil samples were irrigated with deionized water throughout cultivation, and a plate was placed under each pot to collect potential leachate during the experiment. Plants (i.e., roots and shoots) were harvested 7 days after chelate amendments.

2.2. Soil and Plant Analysis

Chemical and physical attributes of the soils are presented in Table 1. Harvested plants were rinsed with deionized water then divided into two parts, the roots and the shoots. Consequently, they were dried at 75°C in an oven for 48 hours then weighted. Plant and soil samples were digested with a solution of 3:1 HNO_3:$HCLO_4$ (v/v) [31]. Concentrations of Pb and Cd were determined using the atomic absorption spectrophotometry method.

2.3. Sequential Extraction of Heavy Metals

Cd and Pb speciation in the soil was performed using sequential extraction by Tsai et al. (1998) [33]. The exchangeable fraction was determined through extraction with 20 ml of 1.0M NH_4OAC at pH 7.0 for 30 minutes at room temperature. The carbonate-associated fraction was determined after extraction with 20 ml of 1.0 M NaOAC adjusted to pH 5.0 with acetic acid for 5.0 hours at room temperature. The Mn oxide fraction was determined after extraction with 20 mL of 0.1 M $NH_2OH{\cdot}HCl$ in 0.1M HNO_3 for 30 minutes. The Fe oxide fraction was determined after extraction with 20 mL of 0.04 M $NH_2OH{\cdot}HCl$ in 25% (v) acetic acid for 6.0 hours in a water bath at 96°C. The organic fraction was determined after extraction with 5 mL of 0.01 M HNO_3 and 10 ml 30% H_2O_2 for 5.0 hours in a water bath at 85°C and followed by 15 ml 3.2 M NH_4OAC in 20% HNO_3 for 30 minutes at room temperature. The residual fraction is the above five fractions subtracted from the total metal content.

2.4. Statistical Analysis

All treatments had three replicates in each experiment. The experiment was carried out in a randomized complete block design. Statistical analysis of the experimental data was performed using statistical software known as SAS. Mean comparisons were done with the Duncan test at a significance level of 0.05. Graphs were drawn with Microsoft Excel software.

Table 1. Basic Chemical and Physical Properties of the Contaminated Soil.

Soil Property	
pH (1:2)	7.95
OM (%)	1.17
EC (dS m^{-1})	1.78
CEC (meq 100 gr^{-1})	24.4
Total-N (%)	0.09
Available-P	77.2
Available-K (mg kg^{-1})	1368
Ca+Mg (meq l^{-1})	14
Ca (meq l^{-1})	7
HCO_3^- (meq l^{-1})	10
Cl (meq l^{-1})	15
Cd (mg kg^{-1})	5
Pb (mg kg^{-1})	1189.8
Texture	Silty Clay

3. RESULTS AND DISCUSSION

3.1. Effect of EDTA and EDDS on Plant Growth

As displayed in Figure 1, application of EDDS and EDTA had inhibitory effects on plant growth for two metals. These two chelating agents reduced dry biomass yield of root and shoot with different degrees when compared with plants in the control. Based on statistical analysis and displayed in Figure 1(a), it was observed that in the case of Cd that although there's an arrangement of descending order for this sequence, it didn't show significant differences when compared to the control. The decreasing sequence of aerial parts of Zea mays compared with the Control was as follows:

EDDS-5 > EDDS-10 > EDTA-5 > EDTA-10

The most considerable reduction in biomass of Zea mays shoots was demonstrated by treatment EDTA-10. Biomass of treatments with chelates showed significant differences compared to the control.

Figure 1(b) depicts when 5 mmol kg^{-1} EDDS was applied. Biomass reduction was not significant but when 10 mmol kg^{-1} EDDS was used it appeared to be of great significance. Employing EDTA in concentrations of 5 and 10 mmol kg^{-1} brought about no results. The decreasing order of Z. mays shoot biomass was as follows:

EDDS-5 > EDTA-5 > EDDS-10 > EDTA-10

They showed no significant difference compared to the control.

Numerous reports suggest that addition of some synthetic chelators does in fact have a significantly detrimental effect on plant growth [12, 26]. Lou et al. (2005) discovered that dry biomass of shoots decreased up to 60% and 52% than in the control for corn, and 76% and 61% for beans, respectively, on the 14th day after application of EDTA and EDDS [15]. This is in agreement with the current study. The severe reduction in growth was attributed to the combination of heavy metal concentration and the addition of chelators that exceeded the capacity of plants to activate defense systems. An example, natural low molecular weight organic acids (NLWOA) may damage plasma membranes which are normally stabilized by Ca^{2+} and Zn^{2+} ions [7], and synthetic chelating agents at high concentrations can also be toxic to plants [15, 23].

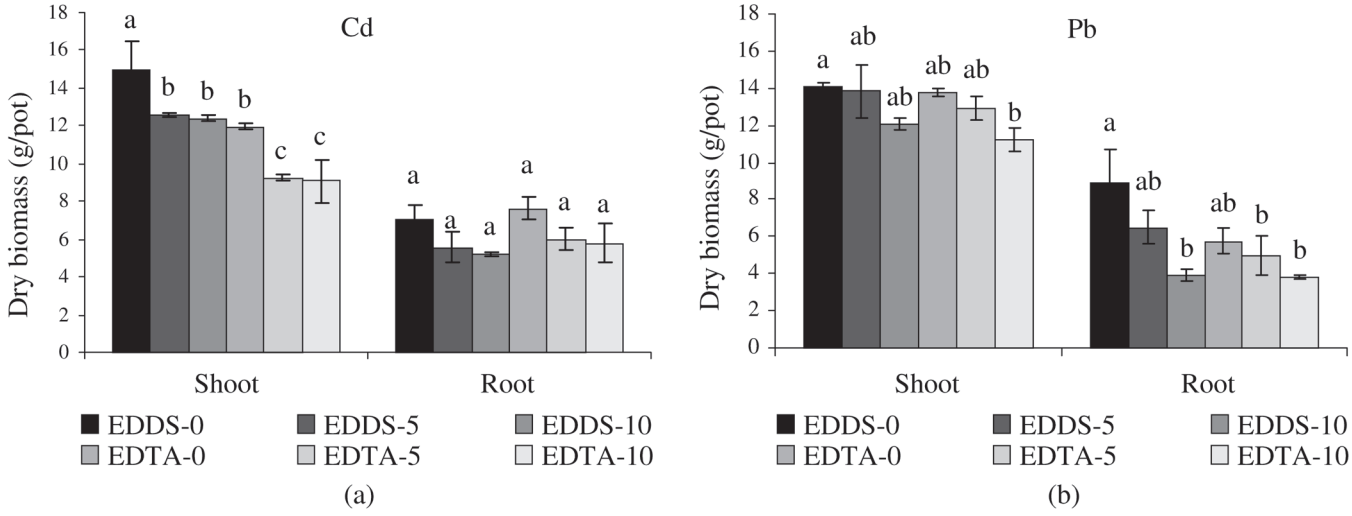

Figure 1. *Dry biomass of Z. mays treated with EDTA and EDDS. The same letters are not significantly different.*

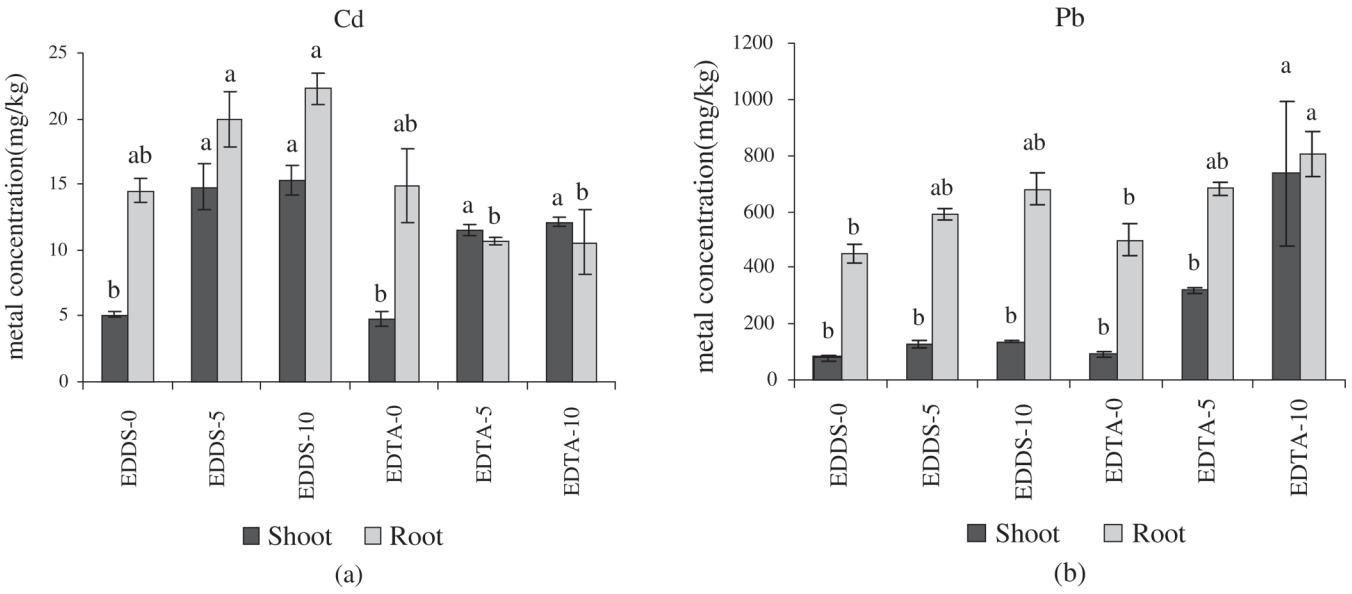

Figure 2. *Effect of EDTA and EDDS on metal concentrations in shoots and roots of Z. mays. The same letters are not significantly different.*

3.2. Effect of EDTA and EDDS on Metal Concentrations of Zea Mays

Addition of EDTA and EDDS to the soil could in fact enhance concentrations of heavy metals in Zea mays' tissues when compared with the control. Also, Huang and Cunningham (1996) found that addition of HEDTA (2.0 g kg^{-1} soil) to a Pb-contaminated soil with total soil Pb at 2500 mg kg^{-1} resulted in a surge of Pb accumulation in corn [6].

As illustrated in Figure 2(a), when EDDS and EDTA were applied at 5 and 10 mmol kg^{-1} Cd concentrations, aerial parts of the plants were significantly increased in comparison to the control. However, for the roots this significant increase was not observed. Furthermore, the differences between chelate levels were insignificant as well. The following treatments of EDDS-5, EDDS-10, EDTA-5, and EDTA-10 increased Cd content in the aerial parts 2.9, 3, 2.4, and 2.5 times higher than of the control, respectively.

Application of EDDS-5 and EDDS-10 lead to a 1.4 and 1.5 times increase in Cd concentrations of the roots when compared to the control. Application of EDTA-5 and EDTA-10 resulted in a decrease of Cd concentrations within the roots, which is consistent with a report from Lou et al. (2006) [16].

In this study, EDDS was remarkably more capacitated than EDTA regarding transportation and accumulation of Cd. The most-impressive treatment was EDDS-10.

As depicted in Figure 2(b), Pb concentrations are enhanced both in the roots as well as in the shoots and is consistent with Xiong et al. (2004) [38]. This enhancement is more remarkable in the roots. This suggests that EDTA was far more efficient in overcoming the diffusion limitation of metals to root surface than the barrier of root to shoot translocation [14].

Under treatments of EDDS-5 and EDDS-10, accumulated Pb in the shoots was 1.6 and 1.8 times higher than in the control. In the roots these concentrations were 1.3 and 1.5 times higher than in the control. Accordingly, treatments of EDTA-5 and EDTA-10 for the shoots resulted in 3.4 and 7.9 times higher concentrations and for the roots 1.4 and 1.6 times higher concentrations compared to that of the control. It is only for the less than 10 mmol kg^{-1} EDTA treatment that Pb concentrations in the roots and in the shoots displayed significant differences when compared to the control and other treatments.

In this study EDTA and in particular EDTA-10 was efficient and capable of Pb accumulation. In chemically enhanced phytoextraction processes, the increased uptake of Pb induced by the application of EDTA can be explained by the effect of improved solubility of Pb and the uptake of the Pb-EDTA complex by plants [29,36]. At the threshold concentration of 5 mmol kg^{-1} of EDTA and above, some chelants including EDTA and EDDS could damage the membrane of root cells which normally function to control uptake and translocation of solutes [36].

3.3. Effect of EDTA and EDDS on Heavy Metal Speciation

Results from sequential extraction are depicted in Figure 3. In treatments, the high proportion (32%) of Cd was associated with the exchangeable fraction which is in agreement with other studies on the high mobility of metals in an acid environment [25]. The decreasing order of different fractions of Cd was as follows:

Exchgeable > Carbonate-associated > Fe-oxide > Residual

The organic-associated and Mn oxide fractions of Cd could not be detected in our study. Most portions (47%) of Pb were observed in the carbonate-associated form which is consistent with a report by Harrison et al. (1981) [4]. The decreasing order of different fractions of Pb was as follows:

Carbonate-associated > Fe-oxide > Organic-associated

The exchangeable fraction of Pb under an ET-10 treatment was much higher than in other treatments. Under no treatments was the Mn oxide fraction of Pb detected. The application of EDTA and EDDS could enhance the exchangeable fraction of heavy metals [31]. Portions of exchangeable Pb and Cd under treatments of ED-5, ED-10, ET-5, and ET-10 were 2.1, 3.1, 5.8, and 12.9 times and 0.98, 1.1, 1.6, and 1.7 times higher than the control, respectively.

It has been reported that accumulation of metals in plants only occurs with high concentrations of metals in soils [25]. EDTA and NLWOA can chelate and mobilize heavy metals in soils and have been used for decontamination or phytoremediation enhancement of metal polluted soils [19]. In response to enhancement of the exchangeable fraction, the residual form of heavy metals was reduced by the addition of EDTA and EDDS [31].

3.4. Bioaccumulation Factor, Transfer Factor and Phytoextraction Efficiency

Bioaccumulation factor (BF) is the ratio of metal concentration in plants to metal concentration in soil. The transfer factor (TF) is defined as the ratio of concentration of metals in stems to that in the roots. These two factors were used to evaluate plant effectiveness regarding metal accumulation and translocation [30]. This ratio higher than one indicates higher concentrations of metals in a plant and is one of the factors indicating suitability of plants for use in phytoremediation [31]. Phytoextraction efficiency of plants depends not only on metal concentration in aboveground biomass, but to a great extent, on the biomass succumb of the plants [10,24]. Therefore, the remediation factor (RF) is defined as the ratio of metal accumulation in shoots to that in soil [30]. Results are displayed in Table 2. Generally BF and TF of treatments are increased via use of chelates. Variations of TF for Pb ranged from 0.17 to 0.91 and for Cd from 0.35 to 1.16. TF for Pb in the treat-

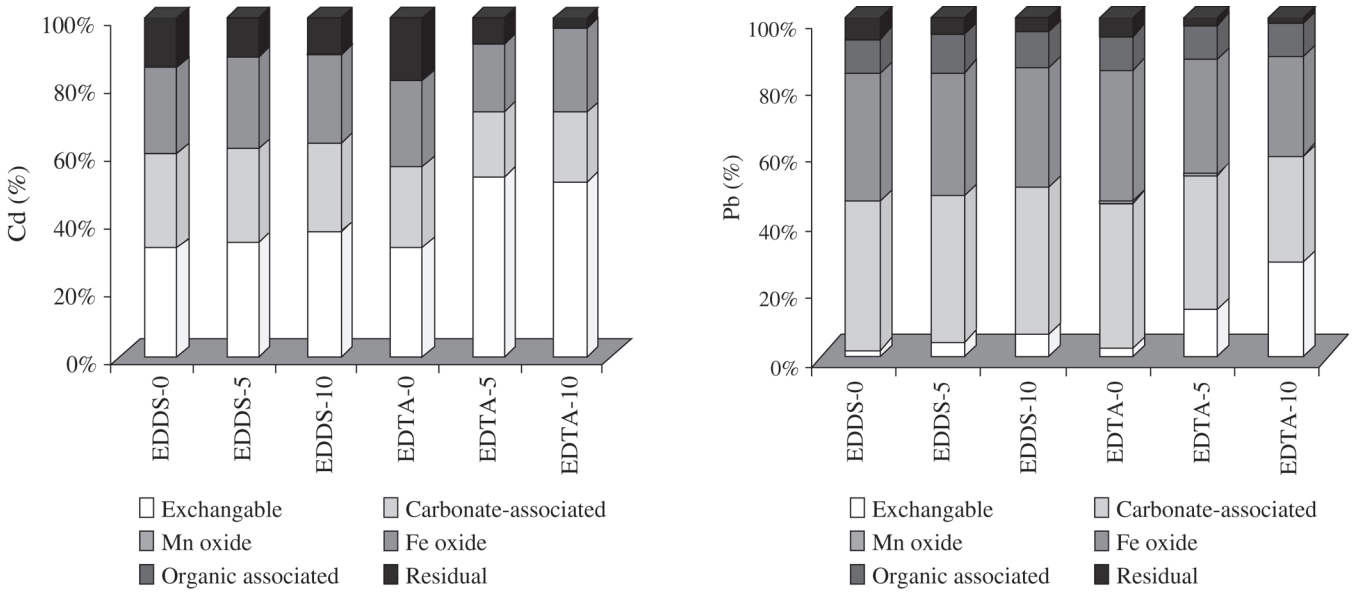

Figure 3. *Effect of EDTA and EDDS on heavy metal speciation.*

ment of EDTA-10 was approximately one and BF exceeded one and in EDTA-5 was close to one. TF of Cd was also close to one in treatments using 5 and 10 mmol kg^{-1} of EDDS. Both in treatment of EDTA for 5 and 10 exceeded one. BF of this metal was larger than one in all treatments. This was observed with RF as well and the overall amount of it was greater for Cd than it was for Pb. The richest owner of RF for Cd at 0.71% was EDDS-10 and for Pb at 0.26% was EDTA-10.

In this study, addition of EDTA-10 and EDDS-10 demonstrated efficient phytoextraction of Pb and Cd from contaminated soils. However, according to reports, Zea Mays' ability of transporting and for amendment of Cd exceeds that of Pb [16].

4. CONCLUSION

Chelate-enhanced phytoextraction is usually performed using mature plants by applying the chelating agent in solution to the soil about one week before harvesting [37]. In this study it was observed that there is a significant capability of Zea mays for up-taking, accumulation, and absorbing of Pb and Cd. Also, Huang and Cunningham (1996) reported that in combination with soil amendment some agronomic crops such as corn might be used for clean-up of Pb-contaminated soil [6]. Supplement of organic chelating agents increased plant accessibility of metals and as witnessed by their presence, accumulation and transport is heightened. Along with these positive effects chelates displayed negative impacts also. An example is that chelating agents caused severe root and stem biomass loss. Moreover, plant growth at treatments using 5 or 10 mmol kg^{-1} EDTA and EDDS had lower biomass compared with control plants. It is widely accepted that metal mobilization and bioavailability can be more easily assessed from sequential extraction data than from total metal concentrations [21]. Sequential extraction carried out in this study demonstrated that application of chelating agents increased the exchangeable fraction of Cd and Pb. Accordingly, zea mays uptake and accumulate higher concentrations of these two metals. Amounts of TF, BF, and RF also increased with enhancement of chelates. This lead RF to be a maximum for Cd for the EDDS-10 treatment and for Pb for EDTA-10 treatments. Finally, Cd and Pb phytoextraction by zea mays proved to be significantly enhanced following treatments of chelates EDDS and EDTA.

Table 2. BFs,TFs and RFs of Heavy Metals.

	Pb			Cd		
Treatment Mg kg^{-1}	**BF**	**TF**	**RF**	**BF**	**TF**	**RF**
EDDS-0	0.54	0.17	0.01	4.90	0.35	0.28
EDDS-5	0.66	0.21	0.02	9.23	0.77	0.70
EDDS-10	0.73	0.20	0.02	9.96	0.69	0.71
EDTA-0	0.59	0.18	0.01	5.43	0.36	0.22
EDTA-5	0.97	0.46	0.05	6.27	1.07	0.43
EDTA-10	1.34	0.91	0.26	5.68	1.16	0.39

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Quantification of Atmospheric Particle Number Concentration for Selected Children Playgrounds: A Case Study in Istanbul

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ABSTRACT: Air pollution is one of the major problems of megacities in the world. Particulate matter, which is the primary component of air pollution, has several adverse effects on environment and humankind. In megacities, many children spend their free time in playgrounds; consequently most of the air pollutants like particulate matter affect urban children. Although particulate matter concentrations may give an idea on the level of air pollution, particle number concentration in ambient air is an important parameter for understanding the short-term effects on the human respiratory system. However, available data on particle number concentrations in various size ranges are not sufficient to clear the issue. In this study, we measured the particle number concentration in the selected playgrounds of a megacity, Istanbul, using a light scattering particle counter. Particle number distributions were determined on 90 days during the spring 2009. Results showed that particles smaller than 2.5 μm were high in number concentrations in the playgrounds, which were close to the major roads, where the traffic was much more dense. Consequently, a few suggestions were made regarding the location of playgrounds in urban areas.

INTRODUCTION

AIR pollution is a significant environmental problem, especially due to its direct health effects. Children's exposure to air pollution is longer than adults' due to relatively more outdoor activities [2, 19]. Particulate matter (PM) in air increases the health risk, and causes aggravated asthma, respiratory symptoms like coughing and painful breathing, chronic bronchitis, decreased lung functions, and even in some cases premature death [10, 20]. There has been a growing concern on PM pollution pointing out the adverse impacts upon human health. Studies were generally focused on determination of mass concentration, elemental composition, and source apportionment of PM [2, 4, 13]. However, it has been suggested that the numbers of fine particles in air was more correlated with health effects than particle mass concentration, when the mass and the number of fine particle concentration were compared [25]. Therefore, particle number and size distribution collectively can be used as an important alternative to understand the effects of PM [3, 22, 23]. On the other hand, Ruuskanen et. al. (2001) noted that both number and mass concentrations must be measured to get an extensive evaluation of air quality. Although most of the research on health effects of PM focused on aerodynamic diameter and mass concentration, chemical composition was the other important parameter to understand the health effects of PM [7].

In epidemiological studies, results show that there were significant relationships between fine particle (< 2.5 μm) concentrations and respiratory and cardiovascular diseases [1, 14, 16]. Fine particles settled in the gas exchange region of the lung, where the air movement was slow, and particles stayed in the area of respiratory bronchioles [6]. Additionally, various studies noted that the adverse health effects caused by number concentrations of ultrafine particles were larger than those of the mass of the fine particles [11, 12]. Traffic related emissions were a major source of both particle number and mass concentration in mega cities [17]. Fine and ultrafine (< 0.1 μm) particles were formed mostly by vehicular exhaust emissions, while course particles could be generated by combustion but mostly natural processes. Most of the suspended PM consisted of coarse particles (90 to 95%), fine particles contributed only 1 to 8% of the total mass, but ultrafine and

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fine particles were greater in terms of numbers [5, 8, 24]. Hence, the size of PM played significant role in terms of health effects of PM. In this sense, Tittarelli et. al. (2008) suggested using the particle counter device to measure the particle number concentration in ambient air. Similarly, this kind of device was utilized in the study to evaluate the particle size distribution and the exposure of children to PM.

METHODOLOGY

Istanbul is the most populated city of Turkey and the fourth in Europe with nearly 4.5% annual population growth rate and more than 12 million residents [18]. It is located in the northwestern part of Turkey, enclosing the Bosphorus strait, which places the city on two continents; Europe and Asia. Air quality of Istanbul has been a primary concern on last decades due to the significant air pollution episodes in 1980s. Vehicular traffic contributes to the substantial part of this air pollution problem in Istanbul.

In this study, 5 different playgrounds were selected due to the distance to dense traffic; *PG-1* was close to a road with heavy traffic, *PG-2* was near the Bosphorus and also a road was passing close to it, *PG-3* was near a high way, *PG-4* was in a recreational area, and *PG-5* was in a residential area. Locations of the playgrounds were shown in Figure 1. Three of these locations were very close to the dense traffic; PG-1, PG-2, and PG-3. The number of vehicles passing on these roads was given in Table 1.

A six-channel particle counter was used in this study.

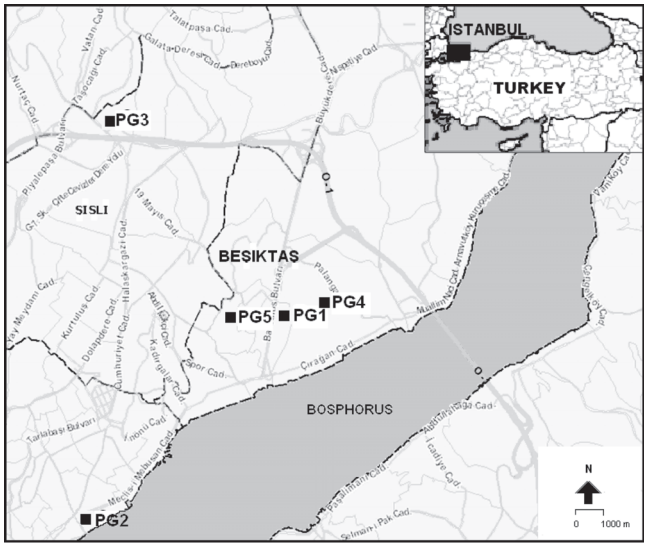


Figure 1. *Locations of selected playgrounds (PG) in Istanbul.*

Table 1. Numbers of Vehicles Passing Near Selected Playgrounds Located Near Heavy Traffic [8].

	Weekdays	Weekend
PG-1	422,994	158,150
PG-2	152,406	59,940
PG-3	18,489	72,645

Device utilized light scattering technology. Sampling flowrate of the instrument was 2.83 $L.min^{-1}$. Average numbers of particles counted in each channel was recorded as daily average values. The particle counter was used for 24 hours continuously and daily calibration was performed once a day. Measurements were carried out from March to the end of the May 2009. Six channels were set to count particles of the following range diameters; 0.30–0.50 μm, 0.50–1.00 μm, 1.00–2.50 μm, 2.50–5.00 μm, 5.00–10.00 μm, and > 10 μm [21, 22].

RESULTS AND DISCUSSION

Measured PM results were summarized in Table 2. Daily variations of the average particle number concentrations were given in the Figures 2 to 7. In Figure 2 and 3, it was seen that PG-3 had the highest particle number concentration between 0.30–0.50 μm, and 0.50–1.00 μm. This result supported the known case that the fine and ultrafine particles were formed mostly by vehicular exhaust emissions. PG-3 was the second playground also in other particle diameter fractions. PG-1 had highest concentrations in Figure 4, 5, and 6. PG-1 was also close to heavy traffic, but sampling location was near the Bosphorus, and therefore this point was affected by sea breeze, reducing the PM concentration in this playground.

PG-4 was expected to have the lowest particle number concentration due to the distance to the dense traffic and also the sampling point was in a recreational area. However, results showed that the lowest particle number concentrations were retrieved in PG-2. Additionally, it could be seen from the figures that the lowest particle size ranges had higher number concentrations [24].

Samples were collected by a low volume sampler in every 6 days of the week at the selected playgrounds. The instrument could only collect one fraction at a time; consequently $PM_{2.5}$ and PM_{10} were collected on two sequential days. $PM_{2.5}$ number concentration was calculated via adding the concentrations in three channels (0.30–0.50 μm, 0.50–1.00 μm, and 1.00–2.50 μm range) [21]. From the Figures 9 to 12, it could be seen

Table 2. Statistical Summary of Measured PM Number Concentrations.

Playground	Size Range	Period (day)	Average PM Number Concentration (number/m^3)	PM Number Concentration Range (number/m^3)	Standard Deviation (number/m^3)
PG1	0.30–0.50 μm	15	1.2E+08	2.9E+07–4.5E+08	1.1E+08
	0.50–1.00 μm	15	1.7E+07	5.5E+06–6.4E+07	1.5E+07
	1.00–2.50 μm	15	1.9E+06	3.4E+05–7.6E+06	2.3E+06
	2.50–5.00 μm	15	7.1E+05	1.3E+05–3.3E+06	9.4E+05
	5.00–10.00 μm	15	1.3E+05	2.6E+04–5.5E+05	1.4E+05
	> 10.00 μm	15	8686	1759–33100	8.2E+03
PG2	0.30–0.50 μm	18	3.4E+07	4.5E+05–7.7E+07	2.5E+07
	0.50–1.00 μm	18	1.0E+06	1.1E+04–3.0E+06	8.9E+05
	1.00–2.50 μm	18	4.9E+04	2.2E+03–1.1E+05	3.0E+04
	2.50–5.00 μm	18	3.7E+04	1.3E+03–6.9E+04	2.4E+04
	5.00–10.00 μm	18	1.1E+04	2.9E+02–2.2E+04	7.1E+03
	> 10.00 μm	18	1144	57–2227	701
PG3	0.30-0.50 μm	17	2.0E+08	1.0E+08–3.2E+08	6.4E+07
	0.50-1.00 μm	17	2.1E+07	1.3E+07–4.4E+07	8.3E+06
	1.00-2.50 μm	17	1.2E+06	7.5E+05–2.1E+06	4.0E+05
	2.50-5.00 μm	17	3.8E+05	2.3E+05–7.2E+05	1.3E+05
	5.00-10.00 μm	17	6.7E+04	3.3E+04–1.8E+05	3.6E+04
	> 10.00 μm	17	4263	1734–12900	2639
PG4	0.30–0.50 μm	8	1.6E+08	7.6E+07–2.6E+08	8.1E+07
	0.50–1.00 μm	8	1.7E+07	6.3E+06–3.3E+07	1.1E+07
	1.00–2.50 μm	8	5.1E+05	2.4E+05–8.4E+05	1.8E+05
	2.50–5.00 μm	8	1.4E+05	6.9E+04–2.4E+05	4.8E+04
	5.00–10.00 μm	8	2.4E+04	1.1E+04–5.1E+04	1.3E+04
	> 10.00 μm	8	1565	495–3643	947
PG5	0.30–0.50 μm	15	9.1E+07	2.3E+07–1.8E+08	5.7E+07
	0.50–1.00 μm	15	6.3E+06	1.9E+06–1.4E+0	73.8E+06
	1.00–2.50 μm	15	5.5E+05	2.1E+05–9.5E+05	2.0E+05
	2.50–5.00 μm	15	2.1E+05	9.3E+04–4.0E+05	7.6E+04
	5.00–10.00 μm	15	4.6E+04	2.3E+04–9.2E+04	1.9E+04
	> 10.00 μm	15	3720	2009–6786	1479

that $PM_{2.5}$, and PM_{10} mass and number trends were generally similar. Mass measurement was performed according to gravimetric method, which the particles are collected on a filter. To measure the PM number concentration light-scattering method was used. Two different measurement methods could be the reason for the incompatible values between mass and number concentrations at some points in Figures 8–12.

Figures showed that PG-1 and PG-3 had the highest particle number concentrations in every measurement range, because these playgrounds were close to the dense traffic. PG-2 had the lowest particle number concentration in each channel due to the effect of sea breeze, although it was close to a road with dense traffic. PG-5 had the stable particle number concentrations at all six measurement ranges. It was expected because

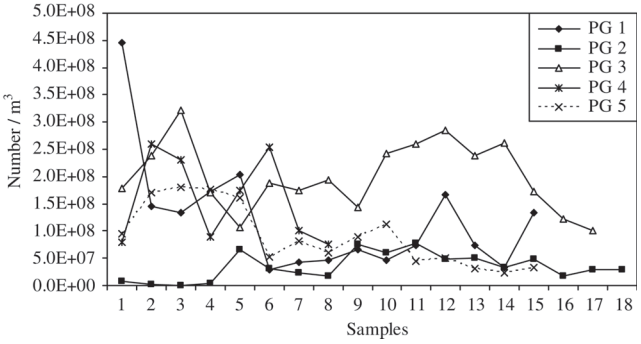


Figure 2. *Particle number concentrations (0.30–0.50 µm).*

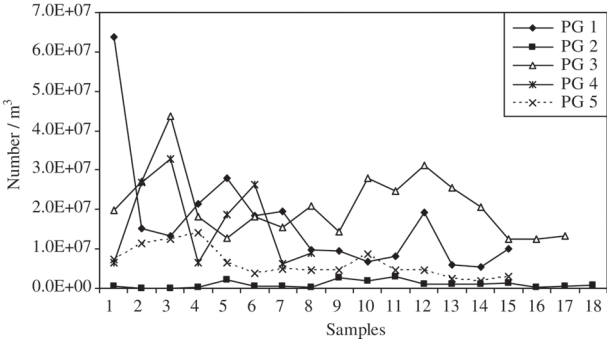


Figure 3. *Particle number concentrations (0.50–1.00 µm).*

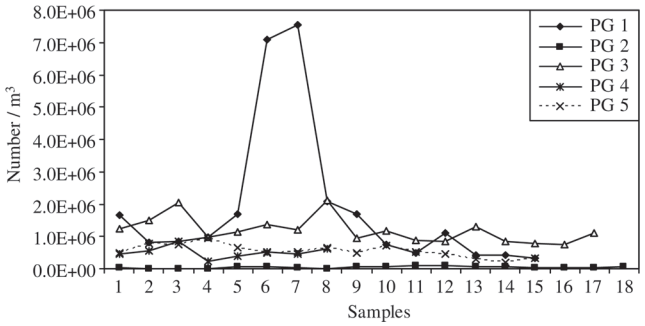


Figure 4. *Particle number concentrations (1.00–2.50 μm).*

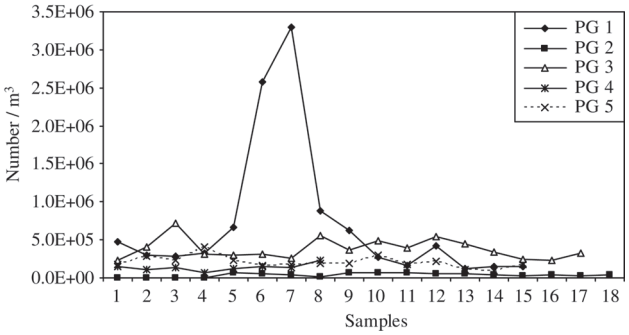


Figure 5. *Particle number concentrations (2.50–5.00 μm).*

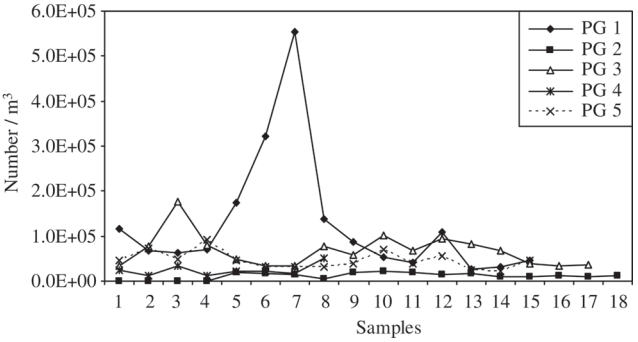


Figure 6. *Particle number concentrations (5.00–10.00 μm).*

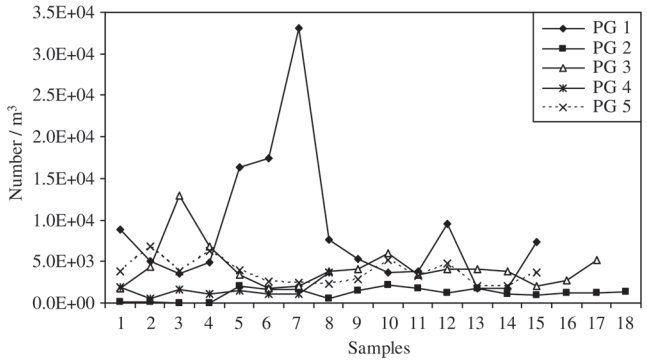
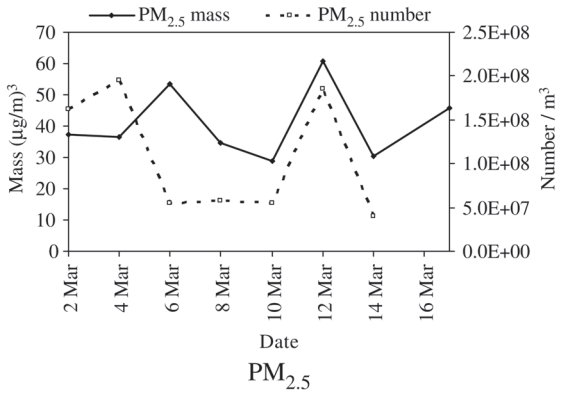


Figure 7. *Particle number concentrations (> 10 μm).*



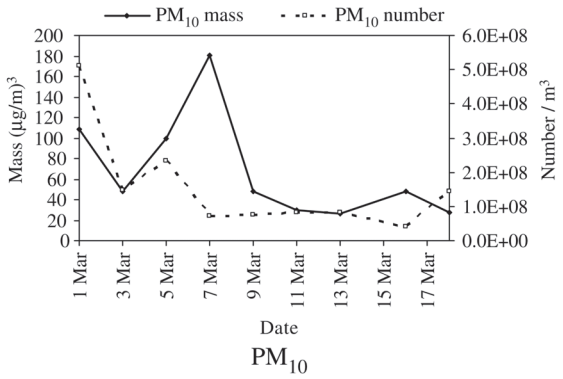
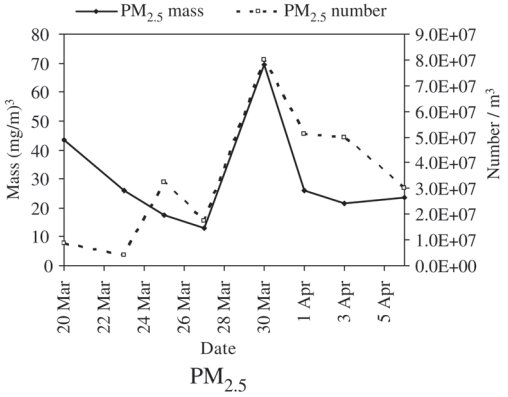


Figure 8. *Mass and number concentrations in PG-1.*



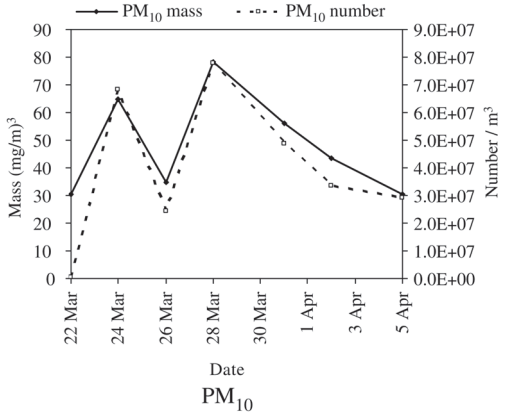


Figure 9. *Mass and number concentrations in PG-2.*

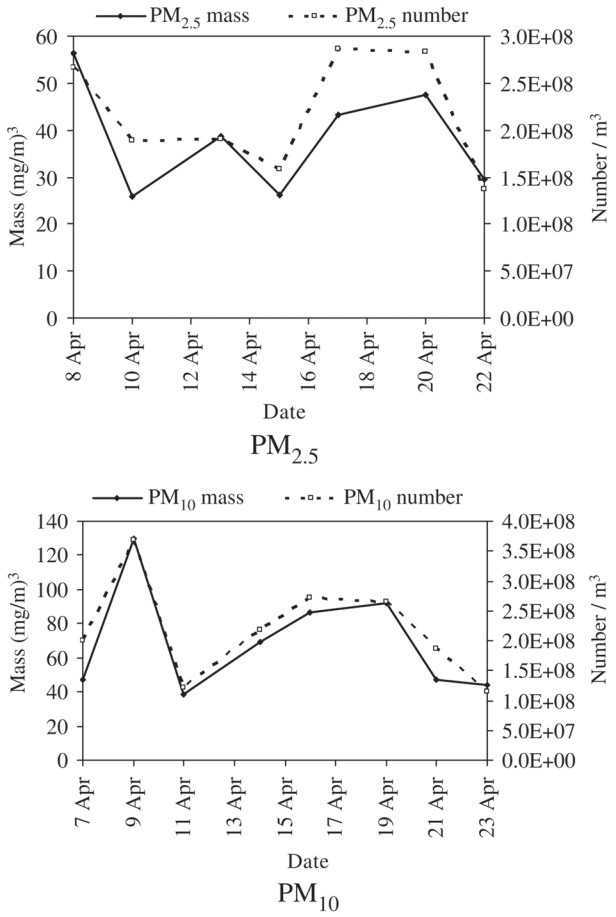


Figure 10. *Mass and number concentrations in PG-3.*

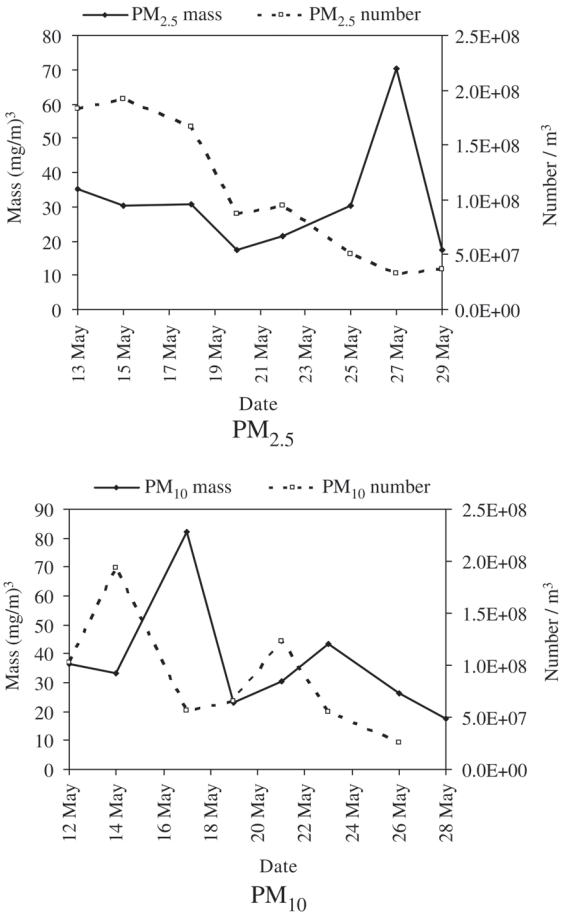


Figure 12. *Mass and number concentrations in PG-5.*

PG-5 was away from the traffic and it was located between the buildings. Thus, convection of the particles was prevented by the buildings, and the lowest concentrations were recorded in this playground.

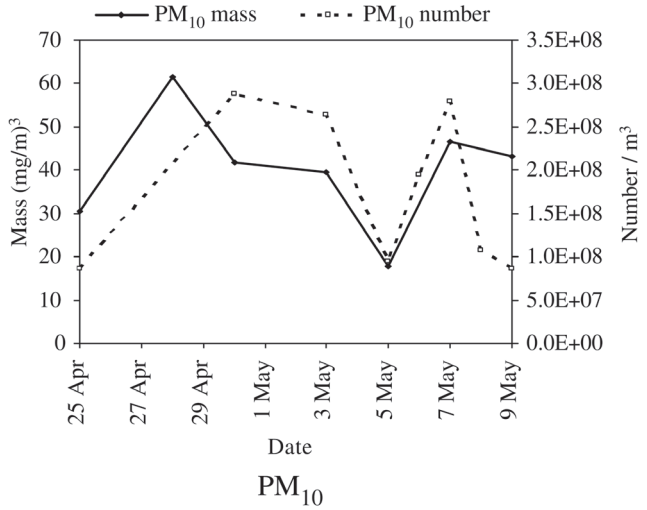


Figure 11. *Mass and number concentrations in PG-4.*

CONCLUSION

Children have higher breathing rates and therefore inhale a relatively larger volume of pollutants than adults, for this reason health implications of near-roadway exposures to vehicle-related pollutants is an important problem for young children. In this sense the results of this study are important for understanding the magnitude of health risks for children playing near the major roadways. Particle number concentrations presented variations between the selected playgrounds in Istanbul. Exposure of children to PM pollution was much more in the playgrounds which were near the highway and dense traffic. Ultrafine and fine particles were high in number concentrations, which pointed out that these particles were formed mostly by vehicular exhaust emissions [24].

Although the ultrafine particles had high numbers in concentration, they contributed to the negligible fraction of the total mass concentration of PM; consequently the mass-based air quality standards did not

best fit the levels of ultra fine particles [15]. Therefore, investigation of particle number concentration was crucial for the assessment of the local air quality. Additionally abatements should be enforced to mitigate the PM pollution in playgrounds. As an initial step, construction of playgrounds near major roadways should be prohibited, and they should be located within a pre-defined distance or farthest from major roadways. And, this strategy should be an integral part of the transportation, and local urban planning.

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Comparison of Specific Nutrients from Activated Sludge and Produced Compost

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ABSTRACT: The objective of this study is to investigate physicochemical characteristics of sewage sludge taken from the municipal wastewater plant of Chotrana in the capital of Tunisia and to investigate compost made from the sludge (15.29 C/N ratios). A comparative study was made for major nutrient elements or macronutrients (e.g., carbon, nitrogen, phosphorous, potassium) and micronutrient elements (e.g., manganese and magnesium). Organic matter concentration at the end of composting was lower in final product. K, Fe, and Mn concentrations were more important in mature compost. Original sludge displays higher C, N, P, Ca, and Mg contents. An important observation was the amount of heavy metals and *E.Coli* present in the final product is lower than amounts mentioned for waste compost used in French norms, suggesting the final compost may be safe for agricultural use as well.

INTRODUCTION

ACCUMULATION of sewage organic wastes is a serious environmental problem in Tunisia. Also, necessity to preserve natural resources and the optimisation of the use of non-renewable energy has encouraged recycling and recovery of these wastes. Among organic wastes recycled in agriculture, residual sludge generated by wastewater treatment is a source of organic matter rich in both phosphorus and nitrogen. It can contribute to rehabilitation of degraded soils by its fertilising and other soil-improving qualities (Martinez et al., 2003a). Recycling of sludge for agricultural purposes seems to be an appealing solution that enables valuable components to be recycled (Dolgen et al., 2006). Sewage sludge has been used as an amendment to agricultural soils (Kidd et al., 2007) and application of sludge also increases soil organic matter content contributing to structural stability of a soil and to its resistance to erosion (Ortiz and Alcañiz, 2006). Composting is a method of waste disposal and producing organic materials may find various applications in agriculture (Haug, 1993). Composting is an aerobic biological decomposition of organic solid substrates, with putrescible materials converted to a stabilised end-product or compost via microbial action. This process is in widely used as a means of treating organic wastes including sewage sludge, animal and agricultural residues, and household refuse (Ndegwa and Thompson, 2001). Through composting, organic matter undergoes partial mineralisation and to a varying degree undergos transformation into humus-like substances. Thus, compost can be used directly in agriculture as an organic amendant to enhance soil tilth and fertility (Sánchez-Monedero et al., 2004). The concept of composting stems not only from the necessity of managing wastes containing considerable amounts of organic matter but also from compulsory rationalization for a fertilizer component, for a organic economy, and from economic reasons. The major nutrient elements or macronutrients in compost include carbon, nitrogen, phosphorous, and potassium. Micronutrient elements mostly used are cobalt, manganese, magnesium, copper, and zinc. Micronutrients are essential for growth and development of microorganisms. Zinc and copper are essential for many microorganisms in the compost while toxic metals are not (Parvaresh et al., 2004). Since microbial activity generates heat and evaporates water, optimal moisture levels need to be established for different composting materials. Optimal moisture levels are usually between 40% and 60% (Bajsa et al., 2003). Temperature should also

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be regulated to ensure both proper oxidation of organic matter into CO_2 and H_2O, and proper hygienization. Once more easily degradable materials have decomposed, compost temperature falls to the ambient temperature level and the process is stabilized. The pH has a marked effect on the microbial population. In the early stages of composting pH is slightly acidic because of the production of organic acids, but later pH increases because of protein decomposition liberating ammonium. The end product has a neutral-alkaline pH. An optimum carbon-to-nitrogen (C:N) ratio should be maintained as microorganisms require C for growth and N for protein synthesis (Bertran et al., 2004). In recent years and with environmental risks from sludge used in agricultural use, more effort was made in Tunisia to develop low cost and high efficient composting technologies for Tunisia.

Accordingly, the objective of this work was to conduct an analytical survey of the physico- chemical features of selected sewage sludge and produced compost.

MATERIALS AND METHODS

Laboratory research was conducted in a laboratory at the Tunis International Center for Environmental Technologies. This work was ongoing in Semptember 2005.

Sampling

Activated sludge was collected from the wastewater treatment plant of Chotrana in Tunis town. This sludge is used in the north of Tunisia as an organic amendment to increase soil fertility. Preservation of sludge was conducted prior to experimental run. Typical characteristics of sludge used in the composting process are displayed in Table 1. A mixture of sludge and greenwaste was composted on a composting platform at 1:1 v/v ratios in periodically overturned outdoor piles, which were sampled for analysis after the beginning of the composting process and monitored over a 90 day period. Compost piles were built following the same protocol and comprised a layer of green wastes (i.e., leaves, grass clippings, trees, and plan/shrub trimmings) followed by a layer of sludge according to experimental design. Progress of composting and microbial activities was evaluated by measuring pile temperature and external temperature during the composting process. The mixture was turned over periodically to ensure aerobic conditions. Numerous samples from various points of the compost heaps were collected. The time selected for sampling was after 90 days. Samples were kept in a deep freeze until analysis. This compost met the French norm on composts made with materials from water treated for pathogenic microorganisms and heavy metals (NF U 44-095). Characteristics of compost are displayed in Table 1.

Table 1. Typical Characteristics of Sludge and the Compost Produced of Charguia (results expressed on dry basis).

Properties	Sludge	Final Compost
OM (g/kg DW)	412	144
C/N (g/kg DW)	7.93	15.29
Cd (mg/kg DW)	180	< 0.6
Cu (mg/kg DW)	23.1	20.6
Hg (mg/kg DW)	0.485	< 0.091
Escherichia Coli (colony forming units g^{-1} fresh material)	$> 1.1 \times 10^6$	9.2×10^2
Thermotolerant coliforms	$> 1.1 \times 10^6$	9.2×10^2

OM: organic matter, TKN: total kjelahl nitrogen, TOC: total organic carbon, Cd: Cadmium, Cu: cuivre, Hg: Mercury.

Physico-chemical Analysis

Nitrogen was determined via the Kjeldahl method (NF ISO 11261) and organic matter was measured by Gravimetry (Rodier 8th edition). Total organic carbon was measured according to the Colorimetry method (ISO 14235). Fe, K_2O, CaO, MgO, and P_2O_5 were analyzed using emission spectrometry-ICP (NF EN ISO 11885). Also, the elements Cd, Cu, and Mn were analyzed by emission spectrometry-ICP (NF EN ISO 11885). Mercury was determined by atomic absorption analysis (NF EN 1483).

Germination Test

Germination tests were performed with (*Helianthus annuus* L). The germination index was determined by placing a layer of compost or sludge sample in a Petri dish covered with a filter paper. Water was subsequently added until the filter paper was completely submerged. Seeds of sunflower (*Helianthus annuus* L) were then rinced many times with distilled water and placed on the filter paper. Percentage of germination was measured after incubating the covered Petri dishes (i.e., three replicates for each sample of compost) in the dark at 25°C for 96 h (Table 2). The germination index (GI) was computed using the following Equation (1):

$$GI = (\text{percentage viable seeds} \times \text{percentage root length})/100 \quad (1)$$

$$\text{Percentage viable seeds} = (\text{number of viable seeds in the sample/in the control}) \times 100 \quad (2)$$

$$\text{Percentage root length} = (\text{root length in the sample/in the control}) \times 100 \quad (3)$$

Statistical Analysis

In order to calculate sample means and standard deviations between the different parameters, all data were statistically analysed using Wessa System Software with a Fujitsu computer. Each sample was considered to be an individual observation. Values are the mean of three independent replicates ±SE (n = 3).

RESULTS AND DISCUSSION

Compost samples used in this study varied in their physical and chemical properties which directly had an affect on compost stability. Mature compost had generally low values for all the parameters determined in this study and was more similar to that of vegetal wastes. However, incorporation of sludge allows for the elimination of two waste materials and ensures a better product is obtained. Use of sludge led to a lower decomposition of lignin and cellulose, perhaps because greenwaste might contain a cellulose fraction bonded to lignin which would make it more difficult to degrade (Paredes et al., 2002). At the end of the experiments, the C/N ratio in mature compost was increased to 15.29 due to removal of carbon as CO_2 upon microbial respiration. Generally, when a waste is composted, there is a decrease in C:N ratio with time due to losses of C as CO_2. Composting of materials with a low C:N ratio results in more N losses than that seen in high C:N ratio wastes (Sanchez-Monedero et al., 2001). Also, microbiological degradation leads to an excess ammonia formation, which increases pH and thereby enhances ammonia volatilization (Andrew et a.,1991). The change observed in the C/N ratio after the composting process is an indicator of the decomposability of a material. Reduction in total organic carbon indicates rapid biodegradation of carbon. The final C/N ratio observed in this study was comparable to that mentioned by Baharuddin et al. (2009). It can be concluded that carbon was not a limiting factor during these composting experiments. Nitrogen loss in the mixture after composting did not exceed 66.7 %. Nitrogen is more difficult to conserve than phosphorus and potash. This is similar to micronutrients, and due to chemical conditions in which they are present they are lost only to leaching. Leaching may leach out the nitrogen but major loss comes from escape of ammonia or other volatile nitrogenous gases from compost materials to the atmosphere. There has been much research and writing regarding conserving nitrogen and other nutrients, particularly with respect to microbiology of the first mixture. Results of investigations and studies on nitrogen utilization in basic biological processes provide fundamental information regarding control of nitrogen loss in composting. Nitrogen loss as ammonia in aerobic composting is affected by the C:N ratio, pH, moisture content, aeration, temperature, and the form of nitrogen compounds at the start of composting (Dolgen et al., 2006; Sanchez-Monedero et al., 2004). High temperatures increase volatilization and escape of ammonia. Since high temperatures are fundamental in aerobic composting and destruction of pathogens, not much can be done about controlling temperatures other than to avoid temperatures which retard bacterial activity and permit ammonia accumulation. Since greatest ammonia loss occurs during early stages of active decomposition, only little conservation of nitrogen will be gained by reducing temperatures after two turns or after the first 6 to 8 days of active decomposition (Paredes et al., 2002). Nitrogen initially present in the material may have an affect on nitrogen conservation. If large amounts of ammonia are present in raw materials, some of this ammonia may be volatilized and lost before organisms have had sufficient time to utilize and stabilize it. This occurs even though the C:N ratio is satisfactory for nitrogen conservation. This can be an important factor since much of nitrogen loss occurs during the first few days of composting. In addition, composts with a high percentage of green cuttings or garden waste (i.e., tree cuttings), under similar conditions for composting, generally give a slightly lower N effect than composts with a high percentage of bio-wastes (Scherer et al., 1996). Since sludge in general has a higher moisture content than is desired in this case, a substance with lower moisture content must be added. Materials utilized for this purpose are called organic amendments or bulking agents. Bulking agents are needed to provide

Table 2. Evolution of Sunflower Germination Parameters in Sludge and Compost Produced (results expressed in %).

	% Viable Seeds	% Root Length	GI[a]
Control	100	100	100
Municipal sludge	65.03 ± 0.31	90.73 ± 0.33	59.00 ± 0.21
Produced compost	80.05 ± 0.07	97.55 ± 0.92	78.08 ± 0.74

All values are reported as mean ± standard deviation between three replicates.
[a]Germination index.

structural support when composting materials are too wet to maintain air spaces within a composting pile and to reduce moisture content and/or to change the C:N ratio. Green wastes (e.g., leaves, grass clippings, trees, and plan/shrub trimmings) were selected as the bulking agent since they are readily available and have good moisture absorption capacity. Therefore, adding bulking agents such as saw green wastes help to reduce the moisture level and to develop the thermophilic condition.

During the study produced compost showed potentially toxic heavy metal contents. However, they were lower than limits established by French legislation (NF U 44-095). Organic matter in the produced compost at 144 g/kg DW was lower compared to the sewage sludge at 412 g/kg DW. Bertoldi et al. (1991) reported that bacteria and pathogenic organisms can generally metabolize readily assimilable organic matter such as alcohols and sugars whereas they cannot multiply on complex compounds such as cellulose, lignin, and humic substances. Benito Marta et al. (2009) demonstrated compositional changes during composting are similar to those observed during natural wood decomposition. Carbohydrates were degraded more rapidly than lignin and the organic matter was degraded relatively nonselectively. Complex organic compounds like lignin are mainly degraded by thermophilic micro-fungi and actinomycetes. During composting, the immature compost material is mineralized and humified into a more complex organic matter. Contreras-Ramos et al (2004) obtained similar results by composting wheat straw with bovine manure. Comparative concentrations of specific nutrients in the sludge and compost are of interest. A high quality compost product was obtained when the compost contains considerable amounts of nutrients and particularly potassium, phosphorus, ferum, calcium, and magnesium. Sludge and sludge compost displayed variability in total C, N, and organic matter as well as P, K, Ca, Fe, Mn, and Mg (Figure 1). Total C content in the sludge decreased in the produced compost. Decrease of C during composting indicates that microorganisms use these compounds as a source to build their own structures and to alter other more resistant carbon fractions. New water soluble carbon compounds of microbial origin may be formed during composting (Charest et al., 2003). Bioavailable C in the first mixture was transformed by microbes into CO_2 and H_2O and resulted in the loss of organic matter that concentrated nutrient contents. A C content could be used to indicate degree of OM degradation that makes up the maturity and stability phases of the compost studied (Benito Marta et al., 2009). Initially, N content in the sludge was at 28.3 g/kg DW. The N content decreased in the final compost to 7.91 g/kg DW. This data demonstrated that the compost obtained did not supply N as effectively as sewage sludge because of greater N losses during composting (Bertran E et al., 2004). In an initial mixture, using organic matter with excess carbon can create problems. To complete the nitrogen cycle and continue decomposition, microbial cells will draw any available nitrogen in proper proportion to make use of available carbon. Total N is affected by the action of proteolytic bacteria and temperature. At high temperatures, N is lost to the atmosphere. It could be necessary to supplement compost with inorganic sources of N depending on soil nutrient status and crop requirements (A. Egrinya Eneji et al., 2001). Archibold (1995) notes N may be one of the most limiting elements for plant growth in the Mediterranean. In mature compost, there is a lower amount of K than in the sludge. Final compost had a high level of K compared with composts found by Cegarra et al. (1993) and Moreno-Caselles et al. (2002) made from municipal solid wastes and manures which are commonly used as organic fertilizers. P and Ca concentrations were lower in the produced compost compared to sewage sludge. Sewage sludge is generally high in Ca since lime is typically added during wastewater treatment. Sewage sludge entering compost composition comes from a sewage plant that applies a dephosphatation process to sewage. Consequently, P is concentrated in residual sludge. Sewage sludges are known to be a source of P for fertilizers (Lisk et al., 1992). Therefore, compost application must be mineralized to release enough available P and Ca which is often a slow process in newly amended soils. Micronutrients are essential for plant regrowth and plant health (McCrimmon, 2002). The final product contains a higher Fe content than sewage sludge. Generally, sludge compost may be no-

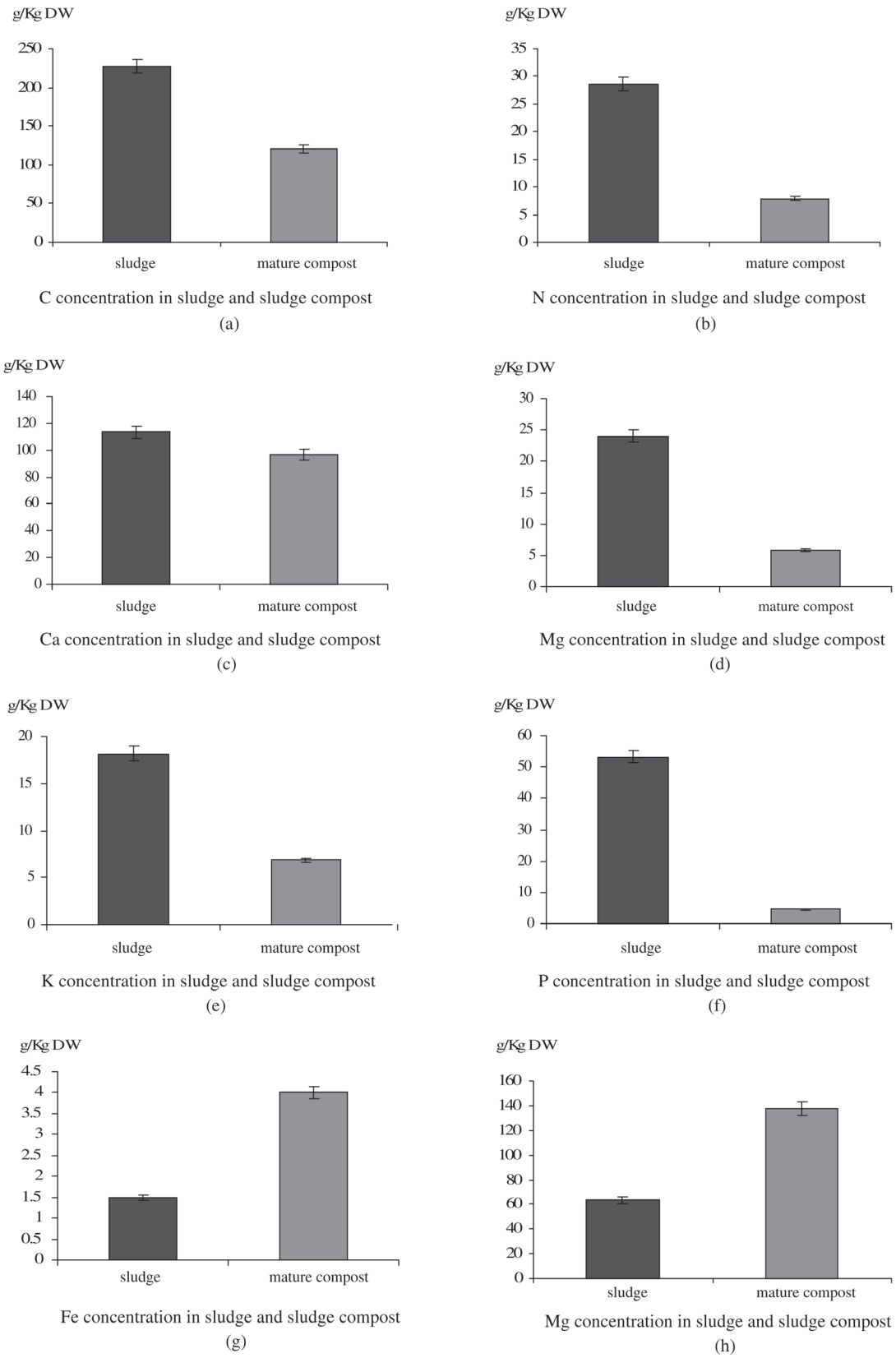

Figure 1. *Concentrations of nutrients in sludge and sludge compost. (a) C concentration in sludge and sludge compost; (b) N concentration in sludge and sludge compost; (c) Ca concentration in sludge and sludge compost; (d) Mg concentration in sludge and sludge compost; (e) K concentration in sludge and sludge compost; (f) P concentration in sludge and sludge compost; (g) Fe concentration in sludge and sludge compost; (h) Mg concentration in sludge and sludge compost.*

tably higher in Fe (Lisk et al., 1992). Cu and Mn were detected in the mature compost as seen in Table 1. It is suggested that most of these micronutrients were derived from organically-bound compounds in the sludge. Also, this compost showed potentially toxic heavy metal contents. The concentrations were lower than limits established by the second draft on Biological Treatment of Biowaste of the European Commission (European Commission, 2001) for compost and stabilised biowaste. Decomposition of organic materials was conducted using microorganisms. In this study, initial total bacteria count was around 1.1×10^6 CFU g^{-1} FW as seen in Table 1. In the final product, it was observed that total bacterial count dropped sharply. This phenomenon might be due to prolonged high temperatures which indirectly inhibit microbial growth. According to Wong et al. (1997), high temperatures will inhibit microbial growth. Moreover, increase of the germination index in the produced product seen in Table 2 suggested composts did not pose any toxic threat to plant growth. Therefore, maturity was sufficient and composting is an important process for eliminating toxic compounds present in sewage sludges (Tiquia et al., 1996). Finally, the produced compost can benefit plant growth and is suitable for agricultural use.

CONCLUSION

This study was a multi-facetted evaluation of the agronomic value of a waste material and the corresponding produced compost. The sludge and corresponding compost varied in composition in terms of nutrients and elemental content. Nutrient elements in the mature compost were generally low compared to native sludge and other mineral fertilizers. Total N, Mg, and P contents were particularly low and were likely to be limiting growth factors for microorganisms or plants because they are essential for plant growth. Hence, N and P are the limiting factors for this sludge compost and to a lesser extent P. Total concentrations of heavy metals in the compost complied with standards established by the second draft on Biological Treatment of Biowaste of the European Commission (European Commission, 2001) making the compost suitable for use as a fertilizer and soil conditioner. The germination index significantly encourages utilisation of mature compost. Longer-term monitoring is required to determine long-term effect of compost types and application rates on plant nutrition and soil characteristics. It can also be concluded the production of compost from sludge and greenwaste is economically justified as it adds value to the economy (i.e., lower cost or price than other compost products on the market) while at the same time it disposes of by-products for agricultural production.

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Augmentation of Protease Production by Supplementing Carbon and Nitrogen Sources into Wastewater Sludge Medium

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ABSTRACT: Production of alkaline protease by *B. licheniformis* ATCC 21424 using wastewater sludge supplemented with different carbon (glucose, maltose, lactose, fructose and starch) and nitrogen (soybean meal, casaamino acids, peptone and $(NH_4)_2SO_4$) sources was investigated to enhance the production. Lactose 1.5% (w/v) as a carbon source and soybean meal 1.5% (w/v) as a nitrogen source has displayed the highest protease production in both shake flask and fermentor among all tested carbon and nitrogen sources. Controlled parameters in the fermentor created a higher total cell count, spore count, μ_{max}, protease activity and specific activity compared to shake flask experiments. Protease activity in the fermentor (15 IU/mL) was 2.6 times higher than that of shake flask (5.7 IU/mL).

INTRODUCTION

PROTEASES are biological catalysts that hydrolyze peptide bonds in the polypeptide chain into lower molecular weight peptides and amino acids and have widely been studied [1]. Proteases are present in all organisms and play important roles in different physiological conditions, catalyzing various metabolic reactions. Among all enzymes, proteases are the most important industrial enzymes accounting for nearly 60% of the industrial market in the world [2]. Alkaline proteases are robust enzymes [3] and they have several considerable and important industrial applications in detergent industry, leather processing, silver recovery, medical purposes, food processing, feeds, meat tendering, brewery and the chemical industry as well as in waste treatment [4, 5]. Alkaline proteases are also capable of contributing to high value added products development because of their characteristic nature of aided digestion [6]. It is a well known fact that the most interesting source of proteases is microorganisms because of their broad biochemical diversity and bioengineering potentiality [7]. One of the largest classes of industrial enzymes is microbial proteases accounting for approximately 40% of the total worldwide enzyme market [8].

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A wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues produce alkaline proteases. *Bacillus* sp. are quite effective producers of extracellular protease among all bacterial species [9, 10]. These enzymes are used as cleansing additives in detergents to facilitate release of proteinacious materials in stains of dirt, blood, milk and more [9].

Fermentation is the process where organisms have been exploited to produce desirable metabolic products including enzymes by offering simulated conditions such as media, temperature, pH, and more. Media rich in complex nitrogen sources can enhance production of proteases under optimum conditoins. Generally, submerged fermentations are in use for production of alkaline proteases due to obvious advantages in consistent enzyme production characteristics with defined medium and process conditions. Solid state fermentation processes have also been studied to a lesser extent and for production of these enzymes [6, 9, 11].

Optimization of medium composition has been carried out to maintain a balance between various medium components, hence minimizing the amount of unutilized components at the end of fermentation. Cost-effectiveness is an important factor to be considered when developing a production medium [9, 2]. Considering the promising applicability of the alkaline protease, it should be produced in high yields using a low-cost medium. This can be achieved by using zero

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cost wastewater sludge as a raw material. Also, it is equally efficient like synthetic medium as already investigated in earlier works [12]. Through wastewater sludge is a good nutrient source for growth of B. *licheniformis* and for enzyme production, because of its complex nature and some non-bioavailable and/or limited carbon availability, sludge may not provide sufficient amounts of readily available nutrients for initial performance of the organism [13]. It is also very well known from the literature that extracellular protease production in microorganisms is significantly influenced by media components, particularly carbon and nitrogen sources [14, 15]. So, supplementation of optimized carbon and nitrogen sources to the sludge medium could further enhance enzyme production.

Protocols optimized for production of protease cannot be extended from one strain to another as the fermentation process. Nutritional requirements are complex and different for each individual strain. Similarly, optimum conditions determined for enzyme production cannot be extended from a semi-synthetic medium to sludge (as a raw material) medium. Therefore, it was necessary to investigate effect of different nutrients (carbon and nitrogen) to find out their sources and their optimum concentrations for maximum protease production employing *B. licheniformis* using wastewater sludge as a raw material.

MATERIALS AND METHODS

Enrichment media and analytical grade chemicals used as reagents for enzyme activity measurements were obtained from Fisher Scientific, Canada. The *B. licheniformis* strain (ATCC 21424) was used in this study. An active culture was maintained by inoculating on nutrient agar plates and incubating at 35°C for 48 hours. Plates were stored at 4°C for later use.

Sludge Samples and Medium Composition

Wastewater secondary sludge samples collected from municipal wastewater treatment plant Communauté Urbaine de Quebec (CUQ, Quebec) were used. Decanted sludge was centrifuged in order to obtain a higher suspended solids concentration of 30 g/L. Then, experiments were conducted at this sludge suspended solids 30 g/L concentration. Physical and chemical characteristics of sludge were measured according standard methods (total solids-2540A, suspended solids-2540D, volatile and volatile suspended solids-2540E, total carbon-5310, total nitrogen-4500-N, ammonical nitrogen-4500-NH_3, total phosphorous- 4500-P and metals-3500) [16].

Inoculum and Cultural Conditions

A loopful of bacteria growth from a nutrient agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of nutrient broth. Before inoculation, nutrient broth was sterilised at 121°C for 15 minutes. The flask was incubated in an incubating shaker (New Brunswick) at 35°C and at 220 rpm for 12 hours. A 2% (v/v) inoculum from this flask was then inoculated into 500 mL Erlenmeyer flasks containing 100 mL of sterilised sludge. Flasks were incubated in the same way for 12 hours. These actively growing cells were used as inoculum for shake flask and fermentor experiments.

Shake Flask Experiments

B. licheniformis strains were tested for extracellular alkaline protease production in shake flasks. A 4.5% (v/v) inoculum was used to inoculate 500 mL Erlenmeyer flasks containing 100 mL sterilised sludge supplemented with different carbon and nitrogen sources [17]. $CaCO_3$ (0.5%) was also added for better control of pH. Flasks were incubated in a shaker incubator (New Brunswick) at 220 rpm for 60 hours at 35ºC. Samples were withdrawn from the Erlenmeyers at every 3 hour intervals for analysis of total cell count, viable spore count, protease activity and specific activity. Specific activity is measured as per total protein.

Carbon Sources

Different carbon sources were tested for alkaline protease production. Sludge was supplemented with final concentration 1% (w/v) of glucose, starch, maltose, fructose and lactose. Different flasks containing sludge supplemented with different carbon sources were sterilized and inoculated with 4.5 % (v/v) of inoculums, separately. Inoculated flasks were incubated at 35°C for 48 hours in a shaker incubator. In order to determine an optimum concentration for lactose, different concentrations of lactose from 0.5%–3% (w/v) were added to sludge and experiments for enzyme production were conducted as mentioned previously.

Nitrogen Sources

Different nitrogen sources were tested for alkaline protease production. Sludge was supplemented with a final concentration of 0.5% (w/v) soybean meal, peptone, casaamino acids and $(NH_4)_2SO_4$. Different flasks containing sludge supplemented with nitrogen sources were sterilized and then inoculated with 4.5% (v/v) of inoculum. Inoculated flasks were incubated at 35°C for 60 hours in a shaker incubator. In order to determine an optimum concentration of soybean meal sludge was fortified with different concentrations of soybean meal (0.5%–3% w/v).

Fermentor Experiments

A fermentor with 7.5 L capacity and 4 L working volume of sludge supplemented with 1.5% soybean meal and 1.5% lactose sterilized at 121°C for 30 minutes was used for extracellular alkaline protease production for comparison with flask level experiments. The medium was inoculated with 4.5% (v/v) inoculum volume. Temperature and pH of the fermentation medium was controlled at 35°C and 7.5, respectively. To maintain dissolved oxygen concentration above 20% saturation (i.e., critical oxygen concentration), the medium was agitated initially at a speed of 200 rpm and finally increased up to 500 rpm. Air flow rate was controlled automatically using a computer controlled system. Samples were withdrawn from the fermentor at 3 hour intervals for analysis of total cell count, viable spore count, protease activity and specific activity.

Analytical

Culture samples withdrawn at 3 hour intervals were analyzed for total cell count and viable spore count as CFU by serial plating technique in nutrient agar plates. Appropriately diluted samples were plated on nutrient agar plates and incubated overnight at 35°C to form fully developed colonies. For viable spore count, appropriately diluted samples were subjected to heat treatment at 80°C for 10 minutes before plating.

Protease Assay

Samples collected during fermentation and after fermentation were centrifuged at 8,000 rpm for 15 minutes at 4°C, and the supernatant was collected as the crude enzyme source. Protease activity in the supernatant was determined according to the modified method of Kunitz [18]. Thus, supernatant obtained was properly diluted with borate buffer at a pH of 8.2. Protease activity was assayed by incubating 1 mL of properly diluted enzyme solution with 5 mL of casein (1.2% w/v, Sigma-Aldrich Canada Inc) for 10 minutes at 37°C in a constant temperature water bath. The reaction was terminated by adding 5 mL of 10% (w/v) trichloroacetic acid. This mixture was incubated for 30 minutes in order to precipitate total non-hydrolysed casein. At the end of the incubation period, samples as well as blanks were filtered using whatman filter paper, 934-AH. Absorbance of the filtrate was measured at 275 nm. Validation of results was established by treating a standard enzyme solution under identical experimental settings where activity was known. One international protease activity unit (IU) was defined as the amount of enzyme preparation required to liberate 1 μmol (181 mg) of tyrosine from casein per minute at pH 8.2 and 37°C. All experiments were conducted in triplicate and mean value was presented.

Protein Estimation

Protein from the enzyme solution was measured by Braford method [19] using bovine serum albumin (BSA) as a standard and with a spectroscopic measurement of the absorbance at 595 nm.

RESULTS AND DISCUSSION

Effect of Carbon Source on Enzyme Production

Different carbon sources such as glucose, maltose, lactose, fructose and starch were tested for alkaline protease production with a sludge medium in shake flask experiments. Composition of sludge is provided in Table 1 and sludge contains nutritients such as C, N, and P as well as other essential elements. Certain metals such as Zn, Mn, and Fe are essential for bacterial growth. Zn stimulates growth of bacteria and Fe plays an important role in the transport of electrons and acts as a cofactor for many enzymatic reactions. Ca and Mg are also vital elements in sludge as Ca stimulates growth of bacteria and Mg acts as an enzymatic regulator [20]. Profiles of total cell count, viable spore count, protease activity and specific activity obtained in shake flask experiments are displayed in Figure 1. Maximum cell count, spore count, protease activity and specific activity occurred at 42 hours for all carbon sources tested. Cells and spores were increased exponentially until 18 hours elapsed and then continued to increase slowly until the

Table 1. Physical and Chemical Characteristics of Secondary Sludge.

Characteristics	Concentration
Physical Characteristics	
Total solids (g/L)	27 ± 1.1
Volatile solids (g/L)	16 ± 0.5
Suspended solids (g/L)	20 ± 0.8
Volatile suspended solids (g/L)	15 ± 0.6
pH	5.6 ± 0.2
Chemical Characteristics	
Total carbon (%, dry total solids)	37.12 ± 1.4
Total nitrogen (%, dry total solids)	5.26 ± 0.2
Ammonical nitrogen (mg N/kg)	675 ± 21.1
Total phosphorus (mg P/kg)	12,322 ± 41.5
Orthophosphates (mg P/kg)	7,760 ± 23
Metals (in mg/kg) (dry basis)	
Al^{3+}	13,501 ± 42
Ca^{2+}	16,100 ± 45.2
Cd^{2+}	3.3 ± 0.14
Cr^{3+}	69.5 ± 2.2
Cu^{2+}	260 ± 8.97
Fe^{2+}	10,495 ± 31.1
Mg^{2+}	1,864 ± 61.5
Mn^{2+}	195 ± 6.75
K^{+}	1,750 ± 55
Pb^{2+}	64 ± 2.3
Zn^{2+}	497 ± 15.8

All experiments were conducted in triplicates and standard errors were always less than ±5%

42 hour mark. Enzyme activity and specific activity increased with fermentation time and then reached a maximum at stationary phase and then declined towards the end of the process (Figure 1). Among all carbon sources, lactose revealed the highest total cell count ($2 \pm 0.1 \times 10^{10}$), spore count ($1.7 \pm 0.08 \times 10^{9}$), maximum specific growth rate (μ_{max}) (0.33 ± 0.013), protease activity (3.8 ± 0.16) and specific activity (2.2 ± 0.1) followed by fructose glucose, maltose and starch (Table 2). Total cell count was higher when μ_{max} values were higher. This is in agreement with results from previous reports [21, 22] that also observed higher μ_{max} values with higher total cell concentration. According to these results, different carbon sources created different final alkaline protease activity at the end of the process. Maximum protease activity was observed when sludge was fortified with lactose (Table 2). Several authors also reported that lactose was a very good carbon source for enhancement of protease activity when supplemented with 4% (w/v) lactose in a basal medium with extracted soyabean cake at 50 g/L, ammonium phosphate at 10 g/L and KCl at 0.3 g/L. Also, it is a good carbon source for enhancement of protease activity when supplemented with and 1% (w/v) lactose in a basal medium with peptone at 10g/L, $(NH_4)_2SO_4$ at 1.0 g/L, KH_2PO_4 at 0.5 g/L, $MgSO_4 \cdot 7H_2O$ at 0.3g/L, $CaCl_2 \cdot 2H_2O$ at 1.0 g/l, NaCl at 1.0 g/L and glycerol at 10 mL/L, respectively [1, 23]. However, Chu [24] reported that lactose had a negative effect on protease yield.. This could be due to the fact that utilization of

Table 2. Extracellular Protease Production of* B. licheniformis *in Shake Flask with Supplementation of Different Carbon Sources and Different Concentrations of Lactose into the Sludge Medium.

Experiment	Maximum Total Count (cfu/mL)	Maximum Spore Count (cfu/mL)	Specific Growth Rate μmax (h^{-1})	Maxiumun Protease Activity (IU/mL)	Soluble Protein (mg/ml)	Maximum Specific Activity (IU/mg)
Shake Flask						
Control	$2.6 \pm 0.12 \times 10^{9}$	$2 \pm 0.09 \times 10^{8}$	0.30 ± 0.012	3.1 ± 0.015	2 ± 0.1	1.6 ± 0.07
Glucose	$1.1 \pm 0.05 \times 10^{10}$	$1.2 \pm 0.06 \times 10^{9}$	0.34 ± 0.014	3.2 ± 0.016	2.3 ± 0.11	1.4 ± 0.05
Fructose	$1.5 \pm 0.07 \times 10^{10}$	$1.4 \pm 0.07 \times 10^{9}$	0.29 ± 0.01	13.4 ± 0.017	2.3 ± 0.11	1.5 ± 0.06
Starch	$8.5 \pm 0.43 \times 10^{8}$	$4 \pm 0.18 \times 10^{7}$	0.17 ± 0.01	1.9 ± 0.09	2.5 ± 0.13	0.8 ± 0.04
Maltose	$2.4 \pm 0.11 \times 10^{9}$	$1.7 \pm 0.07 \times 10^{8}$	0.28 ± 0.01	3 ± 0.13	2.4 ± 0.12	1.3 ± 0.06
Lactose	$2 \pm 0.1 \times 10^{10}$	$1.7 \pm 0.08 \times 10^{9}$	0.33 ± 0.013	3.8 ± 0.16	2.2 ± 0.1	1.72 ± 0.08
Lactose (different concentrations)						
0.5%	$7.8 \pm 0.25 \times 10^{9}$	$3 \pm 0.13 \times 10^{8}$	0.187 ± 0.01	3.4 ± 0.15	2.3 ± 0.11	1.5 ± 0.07
1%	$2 \pm 0.12 \times 10^{10}$	$1.7 \pm 0.07 \times 10^{9}$	0.33 ± 0.01	3.8 ± 0.17	2.2 ± 0.1	1.72 ± 0.08
1.5%	$5.7 \pm 0.22 \times 10^{10}$	$2.9 \pm 0.13 \times 10^{9}$	0.36 ± 0.016	4.7 ± 0.2	2.1 ± 0.1	2.24 ± 0.11
2%	$3 \pm 0.14 \times 10^{10}$	$1.9 \pm 0.08 \times 10^{9}$	0.31 ± 0.013	4.1 ± 0.19	2.3 ± 0.11	1.8 ± 0.09
2.5%	$7.4 \pm 0.3 \times 10^{9}$	$2.6 \pm 0.12 \times 10^{8}$	0.19 ± 0.008	3.3 ± 0.14	2.3 ± 0.12	1.4 ± 0.06
3%	$5.4 \pm 0.25 \times 10^{9}$	$1.4 \pm 0.06 \times 10^{8}$	0.18 ± 0.007	2.8 ± 0.13	2.4 ± 0.13	1.2 ± 0.05

Note: Digits in parenthesis represents incubation time. All incubation times were 42 hours except for the controls that had a 48 hour incubation time. All experiments were conducted in triplicates and standard error was always less than ±5%

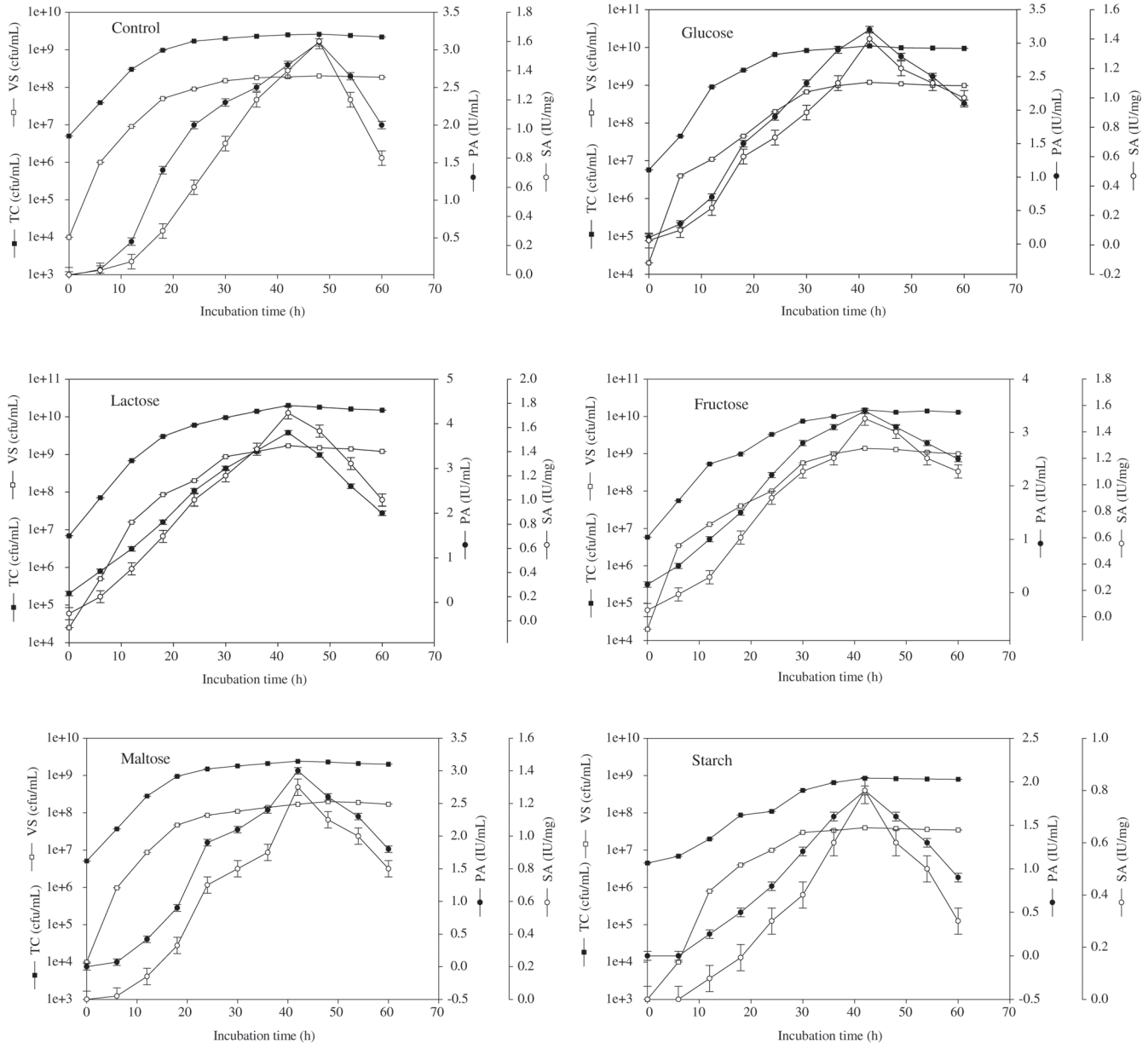

Figure 1. *Effect of different carbon sources on alkaline protease production. (TC = total cell count, VS = viable spore count, PA = protease activity and SA = specific activity).*

different carbon sources varies from microbe to microbe and also because lactose is a suitable carbon source for *B. licheniformis* ATCC 21424 whereas maltose is a suitable carbon source for *Bacillus* sp. APP1 to obtain higher alkaline protease activity. On the other hand, a slight increase in protease activity and specific activity was observed with glucose than control (Table 2), but it supported better growth (i.e., highest maximum specific growth rate and high cell concentration) of bacteria. This is further supported by the findings of Guangrong et al. [25] who stated that simple sugars could be a good carbon source for growth of bacteria. Our results clearly demonstrated that glucose (1%) was not a repressor of protease enzyme for this particular bacterial strain which was in contrast to reports from Rahman et al. [26] and Sen and Satyanarayana [27] who suggested catabolic repression of protease synthesis by glucose. Maltose did not have an impact on protease synthesis, and also there was a 1 log cycle reduction of total cell count compared to lactose. Whereas starch fortification to sludge exhibited a substantial decrease in protease activity, specific protease activity and total cell count (i.e., 2 log cycles reduction compared to lactose). This may be due to the fact that the presence of

starch in the sludge medium might have induced production of amylase instead of protease enzyme. Anbu et al. [8] also found that starch slightly suppressed protease enzyme production from *Shewanella oneidensis* MR-1.

Results obtained on growth and enzyme production by supplementing sludge with different concentrations of lactose (0.5% to 3% w/v) are illustrated in Figure 2. Total cell count, spore count, protease activity and specific activity reached a maximum at 42 hours for all concentrations of lactose. Among all concentrations, total cell count, spore count, μ_{max}, enzyme activity and specific activity were higher at 1.5% (w/v) lactose concentration (Table 2). Other researchers reported that 1% (w/v) lactose in shake flask experiments using *Pseudomonas aeruginosa* strain K at pH 7 and at a temperature of 37°C with 2.61 fold increase in activity [1] and 4% (w/v) lactose in shake flask and fermentor experiments using *B. licheniformis* ATCC 21415 with pH 10 and temperature 37°C [23] produced high enzyme activity (37.8 IU/mL). This could be due to the fact that optimum nutrient (carbon or nitrogen) concentration for higher protease production was different from one microbe to another and also depends on an organism's

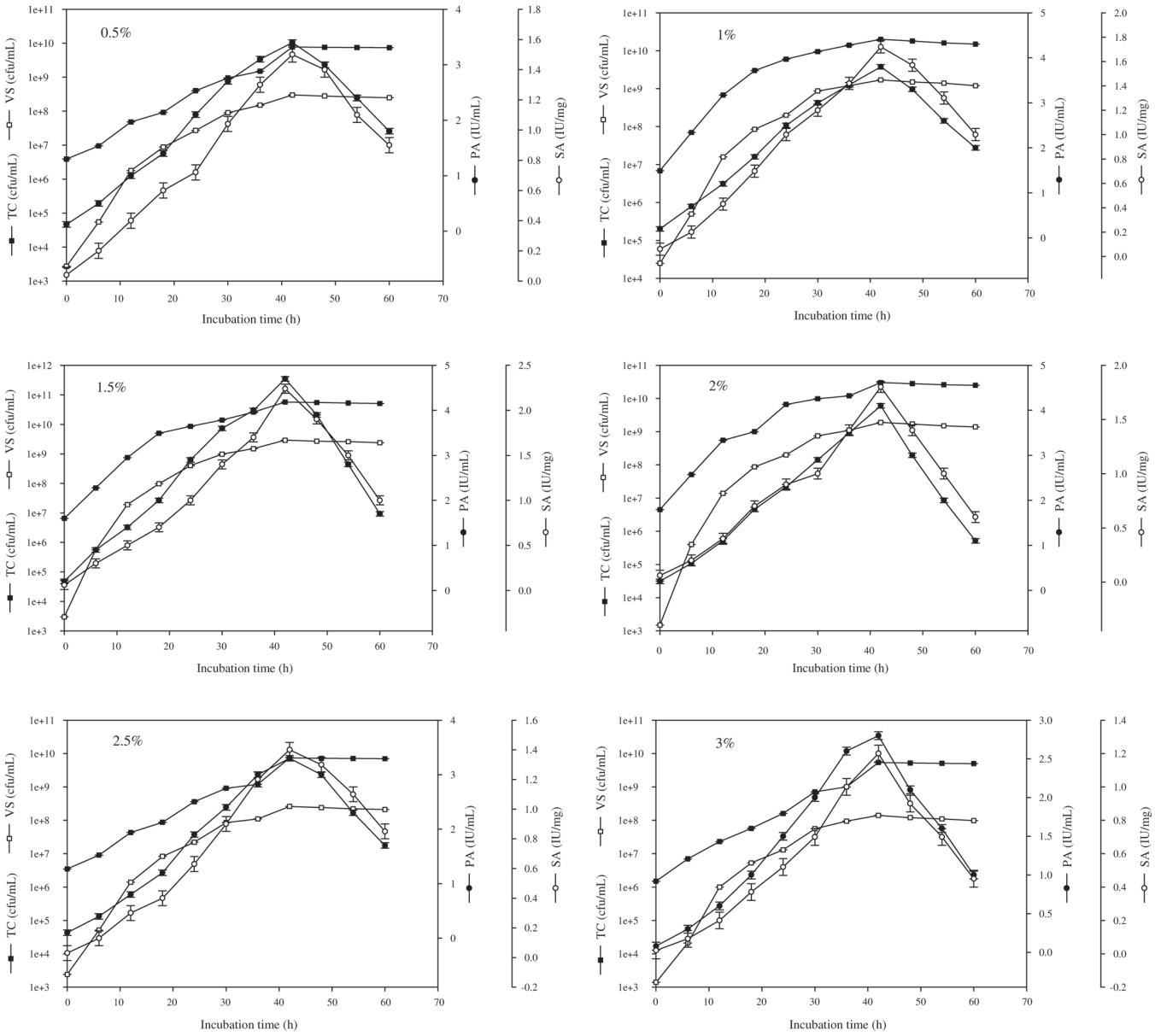

Figure 2. *Effect of different concentrations of lactose on alkaline protease production. (TC = total cell count, VS = viable spore, PA = protease activity and SA = specific activity).*

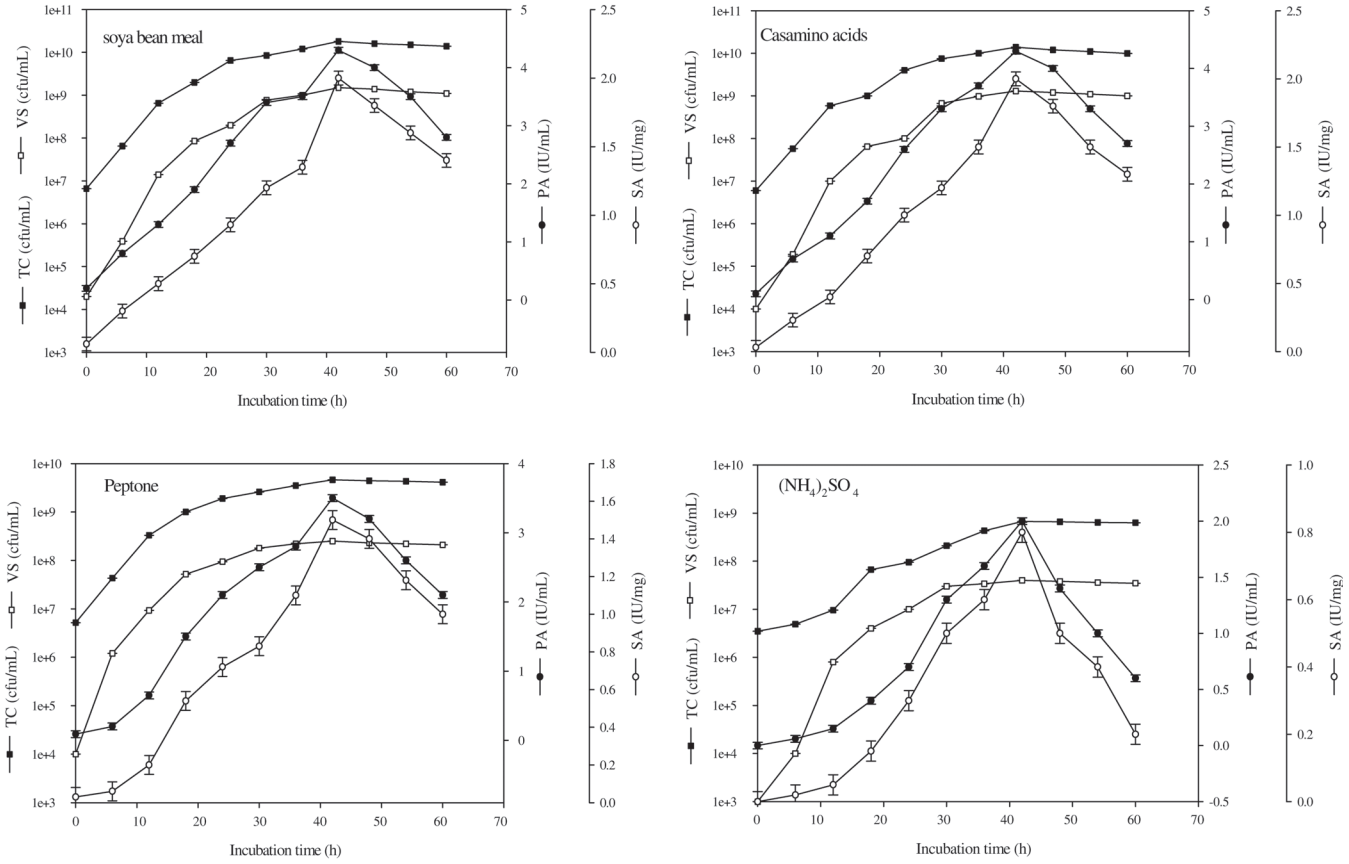

Figure 3. *Effect of different nitrogen sources on alkaline protease production. (TC = total cell count, VS = viable spore, PA = protease activity and SA = specific activity).*

metabolizing capacities [8, 28]. Further, fortification of sludge with 2%, 2.5% and 3% lactose revealed decrease in enzyme activity, respectively (Table 2) and could be attributed to the fact that higher concentration of nutrients might cause inhibition of enzyme synthesis. Protease production is known to be sensitive to repression by higher concentration of readily available carbon sources in the medium. Also, it is supported by other researchers [9, 29].

Effect of Nitrogen Source on Enzyme Production

Results of total cell count, viable spore count, protease activity and specific protease activity obtained when sludge was supplemented with different nitrogen sources are presented in Figure 3. They were found to be higher at 42 hours for all nitrogen sources tested. Viable cells and spores increased exponentially until 18 hours and then continued to increase slowly until 42 hours. Protease activity and specific protease activity increased with fermentation time, attained a maximum at stationary phase and then declined towards the end of fermentation (Figure 3). All nitrogen sources tested had different impacts on enzyme production and results also revealed that complex organic nitrogen sources supported faster growth and higher alkaline protease production than inorganic nitrogen sources. Among all nitrogen sources used, both soybean meal and casaamino acids exhibited significant effect on production of alkaline protease enzyme and they displayed highest cell count, spore count, μ_{max}, protease activity and specific protease activity, respectively (Table 3). Though both soybean meal and casaamino acids had displayed better growth and protease activity, soybean meal was chosen for further study because of its inexpensive and readily available nature. In earlier studies, Bhavna and Gupta [30] also replaced casaamino acids with soybean meal which was 50 times less expensive than casaamino acids. This confirms earlier findings that complex nitrogen sources are needed for better alkaline protease production [1, 6, and 28] and for several other reports on soybean meal as an enhancer for protease production [24, 25, and 31]. Peptone enhanced the growth and protease production to certain extent, but

Table 3. Extracellular Protease Production of B. licheniformis in Shake Flask and Fermentor with Supplementation of Different Nitrogen Sources and Different Concentrations of Soybean Meal.

Experiment	Maximum Total Count (cfu/mL)	Maximum Spore Count (cfu/mL)	Specific Growth Rate μmax (h^{-1})	Maxiumun Protease Activity (IU/mL)	Soluble Protein (mg/ml)	Maximum Specific Activity (IU/mg)
Soyabean meal	$1.8 \pm 0.08 \times 10^{10}$	$1.5 \pm 0.07 \times 10^{9}$	0.33 ± 0.015	4.3 ± 0.2	2.2 ± 0.09	2 ± 0.08
Casaamino acids	$1.4 \pm 0.07 \times 10^{10}$	$1.3 \pm 0.055 \times 10^{9}$	0.30 ± 0.014	4.3 ± 0.21	2.2 ± 0.11	2 ± 0.1
Peptone	$4.6 \pm 0.2 \times 10^{9}$	$2.5 \pm 0.11 \times 10^{8}$	0.28 ± 0.013	3.5 ± 0.17	2.4 ± 0.12	1.5 ± 0.07
$(NH_4)_2SO_4$	$6.7 \pm 0.29 \times 10^{8}$	$4 \pm 0.18 \times 10^{7}$	0.16 ± 0.01	2 ± 0.09	2.5 ± 0.125	0.8 ± 0.03
Soybean Meal Different Concentrations (shake flask)						
0.5%	$1.8 \pm 0.08 \times 10^{10}$	$1.5 \pm 0.08 \times 10^{9}$	0.33 ± 0.017	4.3 ± 0.22	2.1 ± 0.11	2 ± 0.11
1%	$2 \pm 0.09 \times 10^{10}$	$1.9 \pm 0.09 \times 10^{9}$	0.34 ± 0.018	4.5 ± 0.23	2.3 ± 0.12	2 ± 0.09
1.5%	$4.9 \pm 0.22 \times 10^{10}$	$3.2 \pm 0.17 \times 10^{9}$	0.35 ± 0.018	5.2 ± 0.24	2.1 ± 0.1	2.5 ± 0.13
2%	$3.5 \pm 0.16 \times 10^{10}$	$2.5 \pm 0.13 \times 10^{9}$	0.33 ± 0.016	4.7 ± 0.22	2.3 ± 0.115	2 ± 0.12
2.5%	$1.2 \pm 0.06 \times 10^{10}$	$1.1 \pm 0.04 \times 10^{9}$	0.30 ± 0.014	3.3 ± 0.17	2.4 ± 0.13	1.4 ± 0.07
3%	$1.5 \pm 0.07 \times 10^{9}$	$1.3 \pm 0.05 \times 10^{8}$	0.17 ± 0.009	2.5 ± 0.14	2.7 ± 0.14	0.9 ± 0.05
Lactose + soybean meal	$6 \pm 0.28 \times 10^{10}$	$3.7 \pm 0.19 \times 10^{9}$	0.36 ± 0.019	5.7 ± 0.28	2.2 ± 0.11	2.6 ± 0.12
Fermentor						
Control	$6 \pm 0.31 \times 10^{9}$	$5.2 \pm 0.3 \times 10^{8}$	0.38 ± 0.017	11 ± 0.53	1.9 ± 0.1	5.8 ± 0.3
Lactose + soybean meal	$1.5 \pm 0.08 \times 10^{11}$	$1.2 \pm 0.07 \times 10^{10}$	0.40 ± 0.02	15 ± 0.7	1.7 ± 0.09	8.8 ± 0.42

Note: Digits in parenthesis represents incubation time. All incubation times were 42 hours. All experiments were conducted in triplicates and standard error was always less than ±5%.

$(NH_4)_2SO_4$ drastically reduced growth as well as protease enzyme production. This was due to the fact that ammonium sulphate would be attributed to fast release of ammonia in the medium [8, 28, and 32]. The released ammonium ion might interfere with utilization and metabolism of peptides through catabolite repression, consequently suppressing bacterial sporulation and thus reducing alkaline protease production indirectly [33]. On the contrary, Sinha and Satyanarayana [34] suggested alkaline protease production from thermophilic *B. licheniformis* was significantly high with inorganic nitrogen sources such as ammonium sulphate and ammonium nitrate.

Different concentrations of the best nitrogen source (i.e., soybean meal) from 0.5% to 3% (w/v) were supplemented with sludge and subjected to alkaline protease production. Results are displayed in Figure 4 and in Table 3. Sludge fortified with 1.5% (w/v) of soybean meal had produced highest cell count spore count, μ_{max}, alkaline protease and specific activity (Table 3) at 42 hours. Similar findings were reported by Joo et al. [32] who also observed highest protease production (0.64 IU/mL) with 1.5% (w/v) soybean meal concentration supplemented with a medium containing 1% (w/v) casein and 0.5% (w/v) potassium phosphate using *Bacillus horikoshii* in shake flasks at pH 9.0 and temperature of 34°C. The current study is further supported by several other findings. Nilegaonkra et al. [35] reported 1% (w/v) soyabean meal as optimum for maximum enzyme production (0.69 IU/mL) using *Bacillus cereus* MCM B-326 in shake flasks with pH 9 and temperature 30°C. Sutar et al. [36] found 4% (w/v) soybean meal concentration as optimum for protease production (20.1 U/mL) from *Conidiobolus coronatus* in shake flasks at pH 7 and temperature 30°C. This suggests optimum nutrient concentration for high protease production varies from organism to organism due to their different metabolic capacities and composition of medium [6, 8, 28]. Protease production also depends on suitable medium ingredients for the source of carbon and nitrogen, and enzyme production could be increased by adequate concentration of medium ingredients. Likewise, fortification of sludge with suitable nutrients was required because sludge might not provide sufficient amounts of suitable nutrients for optimum growth and protease production or all carbon and nitrogen may not be readily available for growth and enzyme production. It is known that a large part of carbon and nitrogen content of sludge is not available for microbial growth [13].

Validation of Optimal Concentration of Carbon and Nitrogen Sources for Enzyme Production in the Shake Flask and Fermentor

Optimum concentration of 1.5% (w/v) lactose and 1.5% (w/v) soybean meal were supplemented with sludge together in a shake flask to evaluate their effect on enzyme production as well as total cell count, viable

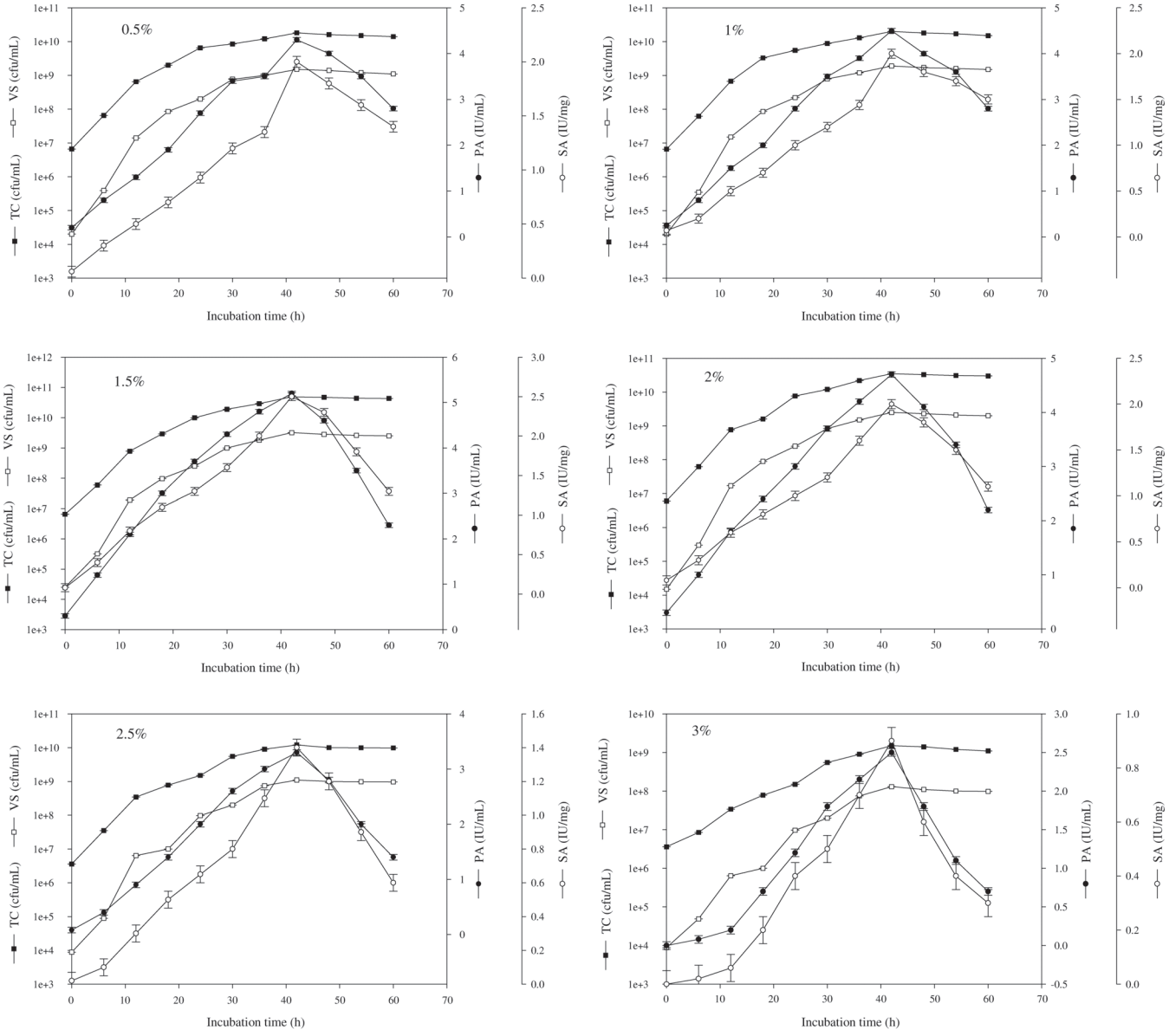

Figure 4. *Effect of different concentrations of soybean meal on protease production (TC = total cell count, VS = viable spore, PA = protease activity and SA = specific activity).*

spore count, protease activity and specific activity. See Figure 5a. Maximum total cell count, viable spore count, μmax, protease activity and specific activity were obtained at 42 hours of fermentation time [Figure 5(a) and Table 3]. Cells and spores were increased exponentially until 18 hours and then they increased slowly and reached a stationary phase. Protease activity and specific activity were increased with fermentation time and reached a maximum in stationary phase. After reaching maximum level, protease activity declined at the end of fermentation. Protease activity and specific activity were highest with lactose at 1.5%, soybean meal at 1.5% and with both lactose plus soybean meal. Thus, sludge required fortification of available carbon and nitrogen in the form of lactose and soyabean meal to enhance growth and protease production.

Validation of optimum concentrations of lactose and soybean meal supplemented together in a sludge medium was conducted in a fermentor. Results of total cell count, viable spore count, protease activity and specific protease activity [Figure 5(b) and Table 3] were at a maximum at 42 hours of fermentation time (stationary growth phase). Protease production was proportional to spore production and protease activity was maximum in the stationary phase of the growth where spore count also reached its maximum value. It could be due to the

reason that when there is a shortage of available aminoacids in the medium, microorganisms start secreting proteases in the medium to degrade complex substrate for cell multiplication and efficient sporulation. There is a correlation between efficiency of sporulation and production of protease. Poorly sporulating cells produce less proteases whereas cells that are highly efficient in forming spores produce high protease [12]. Higher values in the fermentor compared to that shakeflask (Table 3) were due to the fact physical and biological parameters are quite different in the fermentor and shake flasks and could not be controlled properly in the shake flask unlike in the fermentor. Meunier [37] also suggested that proteolytic enzymes were inactivated by a change in pH during growth of *Bacillus* sp. in wastewater sludge shake flask fermentations.

Protease activity (15 IU/mL) and specific activity (8.8 IU/mg) were 2.6 times and 4 times higher, respectively, in the fermentor compared to in the shake flask experiments (5.7 IU/mL and 2.2 IU/mg). This may be due to better control of pH and dissolved oxygen concentration in the fermentor compared to in the shake flask where it is impossible to control parameters properly [21]. Similar observation was made in our earlier work during protease production where protease activity of 11 IU/mL was obtained in a fermentor and 3.1 IU/mL in a shake flask [12]. Yezza et al. [21] also reported two times higher protease activity in a fermentor versus a shake flask during *Bacillus thuringiensis* fermentation of wastewater sludge. Specific protease activity was lower in a shake flask compared to a fermentor. This may be because an organism cannot utilize all soluble protein present in the medium under uncontrolled parameters (i.e., shake flask). Regarding the fermentor, an organism could synthesize protein better under controlled parameters. Also, specific activity varied with different carbon and nitrogen sources (Tables 2 and 3) and may be due to the reason that organism's nutrient (protein) utilization or synthesizing capacities are different.

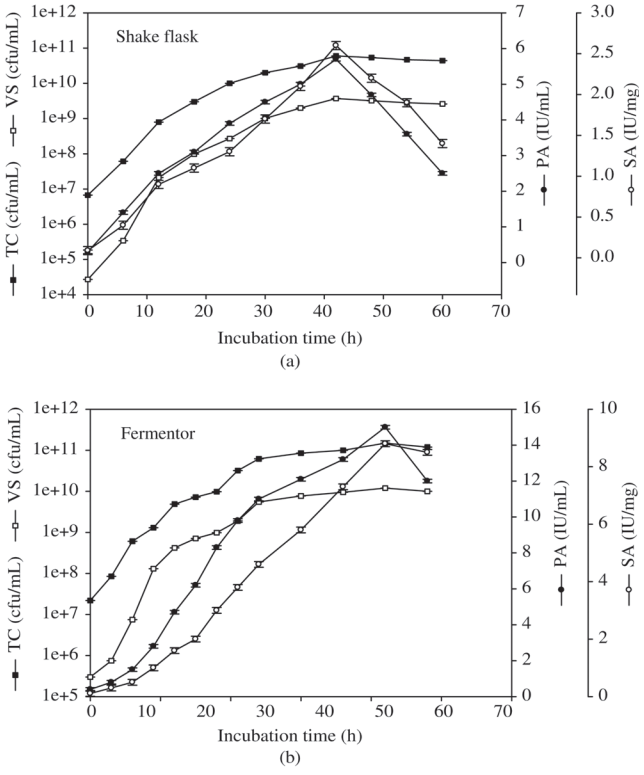

Figure 5. *(a) Validation of optimum carbon and nitrogen sources for alkaline protease production in the shake flask. (TC = total cell count, VS = viable spore, PA = protease activity and SA = specific activity); (b) Validation of optimum carbon and nitrogen sources for alkaline protease production in the fermentor. (TC = total cell count, VS = viable spore, PA = protease activity and SA = specific activity).*

According to results, protease production is directly related to biomass production and corresponds to the stationary growth phase. Protease production and specific activity declined after 42 hours of fermentation time. Protease deactivation could be explained by several reasons. It has been mentioned in many reports that protease synthesis declined when nutrients became limiting. Several other factors like autodigestion of protease and proteolytic attack by other proteases such as intracellular proteases released in the medium after cell lysis may cause protease deactivation [12, 38]. Finally, protease activity declined when protease deactivation was superior to protease production [21]. Results indicated protease production was enhanced from 11 IU/mL to 15 IU/mL when sludge medium was supplemented with lactose and soybean meal as carbon and nitrogen sources in the fermentor. Though sludge contains large amounts of complex carbon and nitrogen content, organisms still require easily available carbon and nitrogen sources for their initial growth which is needed for protease synthesis. So, it is necessary to supplement extra carbon and nitrogen sources to sludge in order to enhance enzyme production.

CONCLUSION

Results from this study suggest that *B. licheniformis* ATCC 21424 produced the highest alkaline protease when wastewater sludge medium was supplemented with extra carbon and nitrogen. This study also suggested that 1.5% lactose as a carbon source and 1.5%

soybean meal as a nitrogen source significantly enhanced alkaline protease production. Protease activity was 2.6 times higher in the fermentor compare to the shake flask. This outcome points towards the possibilities of utilizing other lactose containing industrial waste such as whey powder as a supplement carbon source to be added to the main substrate, wastewater sludge. A cheap nitrogen source, soybean meal can further enhance enzyme yield and therefore the process becomes cost effective and environment friendly.

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Performance of Pulp and Paper Sludge for Reactive Blue 19 Dye Removal from Aqueous Solutions: Isotherm and Kinetic Study

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ABSTRACT: The aim of study is to evaluate performance of dried pulp and paper sludge (DPPS) from Mazandaran wood and paper industries for reactive blue 19 (RB19) dye removal. For this purpose, the effect of various experimental parameters including contact time, pH, adsorbent dose and initial dye concentration were examined. Results showed that an optimum dye removal efficiency (92.27%) was observed at pH = 3, contact time = 30 minutes, adsorbent dose = 6 g/L and initial dye concentration = 100 mg/L. With increase of dye concentration, *Q* (i.e., the amount of removed dye per unit mass of DPPS) increased and reached a maximum value of 33.11 mg dye/g DPPS. The adsorption process follows Temkin isotherm equation (R^2 = 0.992) and the pseudo-second order kinetic model.

INTRODUCTION

DYES are widely used in textile, rubber, paper, plastic and cosmetic industries for coloring purposes [1]. Over 100,000 commercially available dyes exist and more than 7×10^5 tons per year are produced annually [1, 2, 3]. It is considered that generated wastewater from these industries may contain 10–15% of dyes released during the coloring process [4]. Because of growing demand in textiles, the majority of dyes is of synthetic origin and are composed of complex aromatic rings in their structure, which makes them carcinogenic and mutagenic [1, 4]. Therefore, discharge of dye wastewater causes environmental problems [1, 5]. It is necessary to eliminate dyes from wastewater before it is discharged [2]. Different methods including biological and physico-chemical technologies have been used for removing dyes from wastewater such as anaerobic/aerobic treatment, coagulation/flocculation, electro coagulation, flotation, chemical precipitation, photocatalytic decolorization, advanced oxidation and adsorption [6].

Adsorption has been found to be superior to other techniques for dye wastewater treatment in terms of initial cost, simplicity of design, ease of operation and insensitivity to toxic substances. Activated carbon is the most widely used adsorbent because of its high adsorption capacity, high surface area and microporous structure, but it is limited due to its high-cost [7]. It has led to a search for cheaper substitutes [8].

Recently, several studies have been reported using non-conventional low-cost adsorbents including natural materials and waste materials from industry and agriculture [2, 9, 10, 11, 12]. In addition, some investigations have been focused on adsorbents for dye removal like wood sawdust [13], sewage sludge [14], fly ash [15], wheat and rice straw [16, 17], modified basic oxygen furnace slag [18], activated carbons developed from pomegranate peel [8] and so on.

The aim of study is to investigate potential and effectiveness of dried pulp and paper sludge (DPPS) as an alternative adsorbent for removal of reactive blue 19 (RB19) dye. This dye is widely used in textile industries in Iran. For this purpose, the effect of contact time, pH, adsorbent dose and initial dye concentration on the performance of adsorption process were evaluated. Langmuir, Freundlich, Temkin, Harkins–Jura and Dubinin–RadushKevich isotherms were used to de-

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scribe adsorption isotherms. In addition, the pseudo-first order, the pseudo-second order and Elovich model were studied to describe adsorption kinetics.

MATERIAL AND METHODS

Pulp and paper sludge used in this study was collected from Mazandaran wood and paper industries (MWPI) in Mazandaran, Iran. This factory is located in the north of Iran. MWPI is the largest paper manufacture in Iran and produced 100–120 tons of air dried sludge daily. The collected sludge was dried at room temperature in a laboratory for 48 h. For more assurance, it was heated in oven at 50°C for 6 h. The dried pulp and paper sludge (DPPS) was grounded and sieved by 150 μm sieve. The particles less than 150 μm was used in further experiments. Then, DPPS was stored in an airtight glass container. As determined by XRF, chemical composition of DPPS is displayed in Table 1.

Synthetic wastewater was prepared by dissolving reactive blue 19 (Ariazol Brill. Blue R-SP) which was provided by the Alvan Sabet Company, Iran. Chemical structure and general properties of RB19 are presented in Figure 1 and in Table 2. This dye is widely used in textile industries in Iran. A stock dye solution of 1000 mg/L was prepared in distilled water and then diluted to obtain desired concentration.

The solution pH was adjusted with HCl and NaOH. pH measurement was carried out using a 340i/SET pH meter (WTW-Germany). In this study, the effectiveness of different pH (2–12) on the stability and dye adsorption of RB19 was investigated. It was observed that this dye is quite stable in this range. Therefore, the observed dye removal is only a result of the adsorption process. The dye solution and adsorbent was agitated by a jar test at 150 rpm agitation speed. A six beaker jar

Table 1. Chemical Composition of DPPS.*

Chemical Composition	Amount (Wt %)
Al_2O_3	9.7
Fe_2O_3	0.27
CaO	16.4
MgO	1.46
SiO_2	3.8
SO_3	3.52
Na_2O	0.11
K_2O	0.12
P_2O_5	0.2

*Other trace elements analyzed were not reported.
L. O. I = 64.25%.

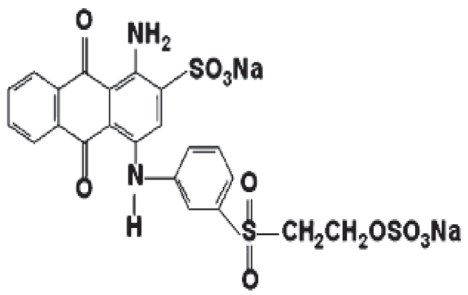

Figure 1. *Chemical structure of RB19.*

test apparatus from Zag-Chemi Company in Iran was used to simulate the adsorption process. Each beaker contained 250 ml of dye solution. All samples were centrifuged at 5,000 rpm for 10 minutes before analysis. A similar method of centrifuging was also used by Tunc study group [1]. Dye concentration was measured using a HACH spectrophotometer (DR/4000) at a wavelength corresponding to the maximum absorbance of 594 nm (λ_{max}) for RB19.

Percentage of dye removal was calculated by the following equation:

$$\text{Dye removal } (\%) = \frac{C_r - C_t}{C_r} \times 100 \qquad (1)$$

where, C_r and C_t are dye concentrations in raw and treated solutions, respectively.

Sorption studies were carried out at 23 ± 1°C. To increase accuracy of the data, each experiment was repeated a minimum of 3 times. Standard divisions from the results were obtained at less than 5% and were displayed in the graphs.

RESULTS AND DISCUSSION

Effect of Contact Time on Dye Adsorption

The relationship between adsorption capacity and contact time was investigated to identify dye removal efficiency. The effect of contact time on RB19 adsorption efficiency is displayed in Figure 2. This step of the

Table 2. Properties of RB19.

Parameters	Values
C. I. name	RB19
Color	Blue
Solubility in water	100 g/L
Empirical formula	$C_{22}H_{16}N_2Na_2S_3$
Formula weight	626.54
λ_{max}	594

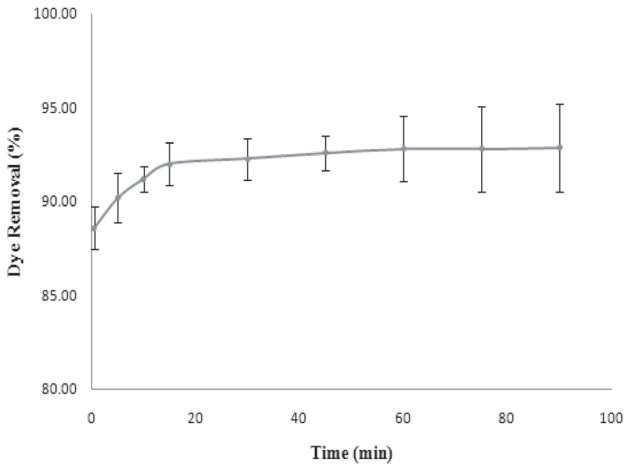

Figure 2. *Effect of contact time on RB19 adsorption by DPPS (adsorbent dose = 6 g/L; initial dye concentration = 100 mg/L; pH = 3; T = 23±1°C; agitation speed = 150 rpm).*

study was accomplished under the following condition: initial dye concentration of 100 mg/L, adsorbent dose of 6 g/L, temperature of 23 ± 1°C, pH of 3 and agitation speed of 150 rpm.

As displayed in Figure 2, adsorption rate initially increased rapidly with increasing contact time and then became constant, approximately. A similar result was presented by other researchers for effect of contact time on adsorption process [19, 20, 21]. Rapid adsorption at the initial contact time is due to availability of the positively charged surface of adsorbent which led to fast electrostatic adsorption of anionic dye from the solution [22]. The later slow rate of dye adsorption is most likely due to electrostatic repulsion between the adsorbed negatively charged adsorbate species onto the surface of adsorbent and the available anionic adsorbate species in solution [22]. It was found that 92.27% removal of RB19 occurred in 30 minutes and after that no significant change was observed. Therefore, because of economical considerations the optimum contact time for the adsorption process was selected as 30 minutes. Other researchers have reported much higher times between 2–20 hr for contact time in comparison with DPPS in the adsorption process [8, 15, 21, 23]. This may be due to special microscopic properties of DPPS. However, contact times less than 2 hours are also reported by other researchers [3, 22].

Effect of pH on Dye Removal

In this step, the behavior of RB19 removal after adsorption by DPPS was studied under various pH values ranging between 2 and 12. Experiments for this step were performed with the following conditions: constant initial concentration is 100 mg/L, adsorbent dose is 6 g/L, temperature is 23 ± 1°C, agitation speed is 150 rpm and contact time is 30 minutes. Results are displayed in Figure 3.

As displayed in Figure 3, dye removal efficiency decreased from 92.27% at pH = 3 to 15.09% at pH = 8 and then increased till pH = 12. Similar results have been reported for adsorption of direct nblue-106 onto activated carbon from an orange peel [22], direct blue 86 onto activated carbon from an orange peel [24] and for adsorption of phenol red onto bottom ash and deoiled soya [25].

Maximum observed adsorption capacity may be due to electrostatic attractions between negatively charged $-SO_3^-$ groups in the dye molecule and positively charged adsorbent surface [22, 25, 26, 27]. With an increase in pH from 8 to 12, dye removal efficiency increased to 65.78%. Lower adsorption at alkaline pH's versus acidic pH's may be due to presence of excess of OH^- ions competing with dye anions for adsorption sites [22]. Also, as mentioned in the materials and methods section, changing pH from 2 to 12 did not have any significant effect on RB19 dye adsorption. A similar result has been reported by Mahmoud study group for RB 19 dye [28]. Therefore, the optimum pH at which the maximum removal occurred was selected as pH = 3.

Effect of DPPS Dose on Dye Removal

In this stage, various amounts of adsorbent ranging

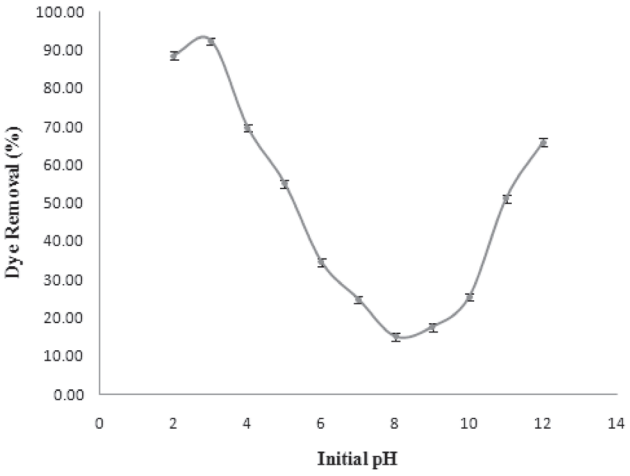

Figure 3. *Effect of pH on removal of RB19 dye by DPPS (adsorbent dose = 6 g/L; initial dye concentration = 100 mg/L; contact time = 30 min;* t *= 23 ± 1°C; agitation speed = 150 rpm).*

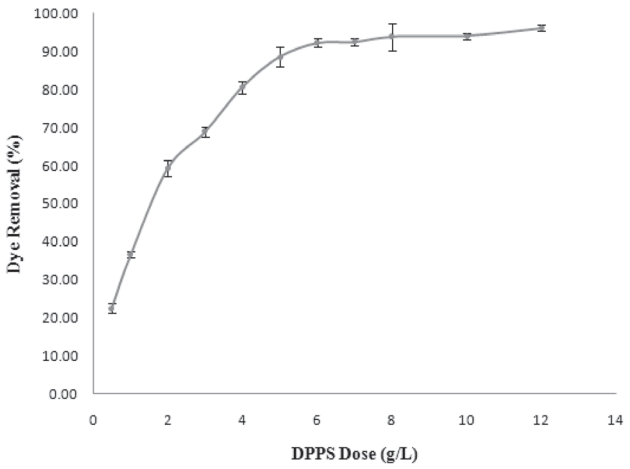

Figure 4. *Effect of adsorbent (DPPS) dose on removal of RB19 dye (pH = 3; initial dye concentration = 100 mg/L; contact time = 30 min; t = 23 ± 1°C; agitation speed = 150 rpm).*

from 0.5 to 12 g/L were studied. For this purpose, initial dye concentration was kept constant at 100 mg/L and pH was adjusted to 3. The effect of adsorbent dose on the adsorption of RB19 by DPPS is presented in Figure 4. As illustrated in Figure 4, dye removal efficiency increased with increase of adsorbent dose. Removal percent of RB19 increased from 22.52% to 92.27% at a dose of adsorbent 0.5–6 g/L and then increased to 96.09% at a dose of DPPS 12 g/L. With increasing amount of adsorbent, the adsorption surface area increases due to greater number of active sites available for adsorption [29]. An amount of 6 g/L was chosen as optimum adsorbent for this study.

Effect of Initial Dye Concentration on Dye Removal

Initial dye concentration is one of the primary factors related to adsorption efficiency. Experiments were performed with variable initial dye concentrations of 25, 50, 75, 100, 150, 200 and 250 mg/L and constant parameters including temperature at 23 ± 1°C, pH at 3, agitation speed at 150 rpm, contact time at 30 minutes and adsorbent dose at 6 g/L. Results are presented in Figure 5. Removal efficiency increased with increase of initial dye concentration from 25 mg/L to 100 mg/L and then decreased. This may be due to saturation of surface area and active sites of adsorbent [30]. However at lower concentrations the dye concentrations to adsorbent sites ratio is higher which causes an increase in dye removal [31]. A similar trend was observed by other researchers for removal of different types of dyes [31, 32].

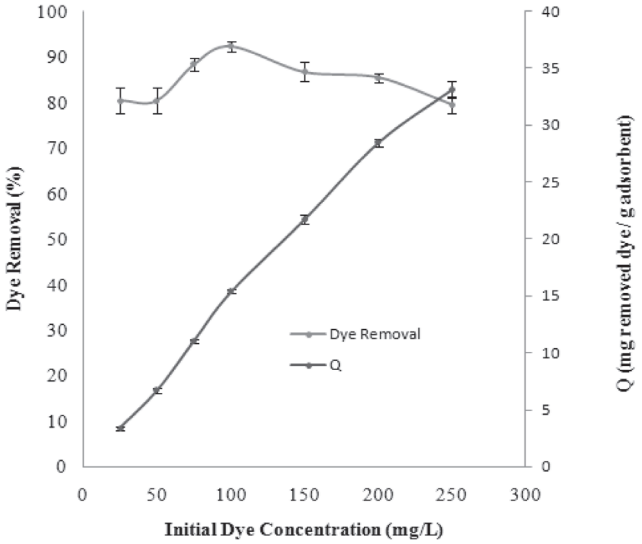

Figure 5. *Effect of initial dye concentration on dye removal efficiency and the amount of the removed dye per unit mass of adsorbent (Q) (pH = 3; adsorbent dose = 6 g/L; contact time = 30 min;* t = *23 ± 1°C; agitation speed = 150 rpm).*

In addition, variations of the amount of dye removed per unit mass of adsorbent (Q) versus the initial dye concentration are displayed in Figure 5. It is observed that Q increased with an increase of dye concentration and ranged from 3.35 to 33.11 mg removed dye/g DPPS. Similar results were presented by other researchers for dye removal [32, 33].

Adsorption Isotherms Study

In order to obtain an interaction between the adsorbent and adsorbate, adsorption isotherm studies were conducted with different adsorbent doses ranging from 0.5 to 12 g/L at pH = 3 and initial dye concentration = 100 mg/L. Five isotherms including Langmuir, Freundlich, Temkin, Harkins–Jura and Dubinin–RadushKevich were tested in this study which are explained below.

Langmuir isotherm: The Langmuir model describes that uptake of dye molecules occurs on a completely homogenous surface by monolayer adsorption with negligible interaction between adsorbed molecules [8, 19, 34]. The Langmuir equation is presented in the linear from as follows [8]:

$$\frac{C_e}{q_e} = \frac{1}{K_L Q_m} + \frac{C_e}{Q_m} \qquad (2)$$

where q_e and q_t refer to the amount of dye adsorbed (mg/g) at equilibrium and at t (min), respectively. C_e is

concentration of dye at equilibrium (mg/L). K_L is the Langmuir adsorption constant (L/mg) and Q_m is the theoretical maximum adsorption capacity (mg/g).

Freundlich isotherm: The Freundlich isotherm describes that the uptake of dye molecules occurs on a heterogeneous surface by monolayer adsorption [19]. The Freundlich equation is presented in the linear from as follows [8]:

$$\log q_e = \log K_F + \frac{1}{n}\log C_e \quad (3)$$

where K_F (L/mg) and n are isotherm constants indicating capacity and intensity of adsorption, respectively.

Temkin isotherm: Temkin and Pyzhev studied heat of adsorption and the adsorbent-adsorbate interaction on the surface [20]. The Temkin isotherm equation is given as [8, 20]:

$$q_e = B_1 \ln K_T + B_1 \ln C_e \quad (4)$$

where $B_1 = RT/b$, T is the absolute temperature in K and R is the universal gas constant, 8.314 J/mol. K_T is the equilibrium binding constant (L/mg) and B_1 is related to heat of adsorption.

Harkins–Jura isotherm: The Harkins–Jura adsorption isotherm is expressed as [8]:

$$\left(\frac{1}{q_e^2}\right) = \left(\frac{B_2}{A}\right) - \left(\frac{1}{A}\right)\log C_e \quad (5)$$

where B_2 and A are isotherm constants. The Harkins–Jura adsorption isotherm relates to multilayer adsorption and can be explained with the existence of a heterogeneous pore distribution [8].

Dubinin–RadushKevich isotherm: The linear form of the Dubinin–RadushKevich isotherm equation can be expressed as [8]:

$$\ln q_e = \ln Qs - B\varepsilon^2 \quad (6)$$

where Qs is the theoretical monolayer saturation capacity (mg/g), B is the Dubinin–Radushkevich model constant (mol^2/kJ^2). ε, is the polanyi potential and is equal to [8]:

$$\varepsilon = RT\left(1 + \frac{1}{Ce}\right) \quad (7)$$

Isotherm constants and correlation coefficient (R^2) are presented in Table 3. Results displayed that the Temkin adsorption isotherm was the best model for dye adsorption on DPPS with R^2 of 0.992.

Table 3. Adsorption Isotherms Constants.

Isotherm Type	Isotherm Constants	R^2
Langmuir	K_L = 0.028 (L/mg)	0.921
Freundlich	K_F = 3.58 (L/mg), n = 1.529	0.912
Temkin	K_T = 0.319 (L/mg), B_1 = 16.1	0.992
Harkins–Jura	A = 111.11, B_2 = 1.67	0.515
Dubinin–RadushKevich	B = 0.775 (mol^2/kJ^2)	0.977

Adsorption Kinetics Study

In order to investigate the mechanism of sorption, the kinetics parameters for the adsorption process were observed for contact times ranging from 1 to 90 minutes by monitoring percent dye removal. 90 minutes were used for the adsorption kinetic study in order to have more accurate data. Differences between optimum contact and kinetic study times are also reported by other researchers [8, 19]. Kinetics models included the pseudo-first order, the pseudo-second order and the Elovich model in this study.

The pseudo-first order equation of Lagergren is expressed as follows [8, 20]:

$$\log(q_e - q_t) = \log(q_e) - \frac{k_1}{2.303t} \quad (8)$$

where q_e and q_t are the sorption capacities at equilibrium and at time t, respectively (mg/g) and k_1 (min^{-1}) is the rate constant. The pseudo-second order model is presented by the following equation [20]:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e}t \quad (6)$$

K_2 is the rate constant for pseudo-second order sorption (g/mg.min). The simple Elovich model equation is expressed by the following equation [8]:

$$q_1 = a + b\ln t \quad (7)$$

The slope and intercept of plot of q vs. ln(t) were used to calculate values for the constants a and b. Kinetics constants for this study are presented in Table 4. Results

Table 4. Adsorption Isotherms Constants.

Kinetic Type	Isotherm Constants	R^2
Pseudo-first order model	K_1 = 0.0414 (min^{-1})	0.913
Pseudo-second order model	K_2 = 0.441 (g/mg·min)	1
Elovich model	a = 14.94, b = 0.162	0.953

demonstrated that the adsorption process follows a pseudo-second order model.

CONCLUSION

Pulp and paper sludge can be considered as an efficient, abundant, natural and low-cost adsorbent for removal of reactive blue 19 dye from aqueous solutions. To summarize, optimum adsorption of RB19 was obtained after 30 minutes and at this time dye removal percent was 92.27%. Efficiency of DPPS in removal of RB19 was absolutely affected by pH. Maximum removal efficiency was 92.27% at a pH of 3. Removal efficiency increased at the higher dose of adsorbent. Maximum dye removal efficiency (96.09%) was observed at adsorbent dose of 12 g/L (pH = 3). When increase of initial dye concentration occurred the removal efficiency initially increased and then declined. Minimum and maximum removal efficiency was obtained 79.46% and 92.27% and at an initial dye concentration of 100 mg/L and 250 mg/L, respectively. However, Q increased with the increase of dye concentration to 33.11 mg removed dye/g DPPS. The Temkin adsorption isotherm was the best model for RB19 dye adsorption on DPPS with a correlation coefficient of 0.992. Adsorption kinetics were found to follow a pseudo-second-order rate kinetic model, with a correlation coefficient of 1. Based on these results, pulp and paper sludge should be considered as an effective, readily available, natural and low-cost adsorbent solution when removing reactive blue 19 dye from aqueous solutions.

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Aerobic Granular Sludge Cultivated in Modified UASB for the Degradation of Pollutants in Leachate

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ABSTRACT: Formation and performances of aerobic granule sludge were investigated in a modified UASB (MUASB) reactor treating both landfill leachate and domestic wastewaters. Results showed that granules could be formed from flocculating sludge in MUASB within 9 days. Granules had an average diameter of 0.5 mm during steady state operation. Under the averaged influent chemical oxygen demand (COD) and ammonia nitrogen concentration of 2,432 mg/L and 197/L condition, removal of COD and ammonia nitrogen were over 96% and 75%, respectively. These results suggested that conventional UASB could be modified to MUASB aiming to cultivate aerobic granules, by taking off gas-liquid-solid separator on the top and putting a fine-bubble diffuser at the bottom of the reactor. As for mature granules, confocal laser scanning microscopy (CLSM) observation revealed that a lot of filamentous bacteria were presented in the fringe region, while cocci were predominant and formed a relatively condensed region in the granule interior. Proteins and lipids were distributed throughout the granules, whereas α-polysaccharides were mainly distributed in the core of granules. Few β-polysaccharides and dead cells were detected in the granules.

1. INTRODUCTION

GRANULAR sludge was first found in strictly anaerobic systems, such as upflow anaerobic sludge blanket (UASB) reactors, biofilm airlift reactors and anaerobic sequencing batch reactors (SBRs) (Cassidya and Belia, 2005). Developed in recent decades, aerobic granules are novel kinds of compact self-aggregates created by aerobically grown microorganisms, which can be used for the treatment of industrial and municipal wastewater (Liu *et al.*, 2006). Aerobic granular sludge technology is characterized by the higher biomass concentration in the reactors, the coexistence of a heterogeneous biomass and lower sludge production due to high biomass retention. Since the separation of sludge and effluent occurs in the reactors without any supplementary sedimentation tank, very small footprints are required (Alessandra *et al.*, 2009). Due to unique granule attributes, aerobic granulation technology was recently developed to treat wastewaters containing high strength of organics, nitrogen, phosphorus, toxic substances and xenobiotics (Adav *et al.*, 2008 and 2009)

Aerobic granules sludge was usually cultivated and studied from SBR reactor or alternation of Aerobic/Anaerobic Process (AAA), sequencing batch airlift reactor (SBAR). However, no modified UASB reactor has been used so far. It was generally regarded that in UASB technology, the upflow velocity creates a choose press for organic material. Organic material was either eluted or tangled into granule which was easy to precipitate. Thus, it is feasible to utilize UASB reactor for cultivated aerobic granule sludge.

Landfills are prevalent worldwide as a management approach of municipal solid waste (MSW) due to simple operations and low cost. Nevertheless, a landfill generates heavily polluted leachate with significant variations in both volumetric flow and chemical composition. Landfill leachate may contain large amounts of organic matter, ammonia-nitrogen, heavy metals, chlorinated organic and inorganic salts. Removal of organic material based on COD, biological oxygen demand (BOD) and ammonium from leachate is the usual prerequisite before discharging it into natural waters. As a conventional landfill leachate treatment method, biological treatment (suspended/attached growth) is commonly used for removal of the bulk of leachate containing high concentrations of BOD due to its reliability, simplicity and high cost-effectiveness.

Since extracellular polymeric substances (EPS) are

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major components of granules, they are hypothesized to play a central role in the biogranulation process (Liu *et al.*, 2004). Chen *et al.* (2007) developed a six-fold fluorescence staining scheme that simultaneously probed proteins, α-polysaccharides, β-polysaccharides, lipids, total cells and dead cells in aerobic granules.

The aim of this study was to investigate the formation and performance of granular sludge in MUASB reactor treating landfill leachate. Granules were formed from flocculating seed sludge taken from Neijiang sewage treatment plants. According to the Standard for Pollution Control on the Landfill Sit in China (GB16889-2008), leachate of existing landfills could be sent to wastewater treatment plants for further treatment together with wastewater under some conditions. Therefore, leachate is presently treated with sewage in many cities of China. In view of this, the experiment influent in this study was a mixture of leachate and sewage. Results demonstrated that aerobic granule could be cultivated in MUASB reactor treating municipal wastewater and landfill leachate, and organic material and ammonia nitrogen could be effectively removed from landfill leachate with aerobic granule sludge. The spatial distribution of EPS was depicted by CLSM.

2. MATERIALS AND METHODS

2.1 Experimental Installation

MUASB reactor (Figure 1) was used to cultivate aerobic granule sludge. The UASB consisted of a rounded-bottom glass vessel with a height of 200 cm and an inside diameter of 8 cm. The reactor working volume was 8.0 L. The UASB reactor was modified by taking off gas-liquid-solid separator on the top and putting a fine-bubble diffuser at the bottom of the reactor. The influent wastewater was introduced into the bottom of the reactor by peristaltic pump. Sampling ports were available in the side of reactor with interval of 10 cm. Effluent was 30 cm distanced from the bottom of the reactor.

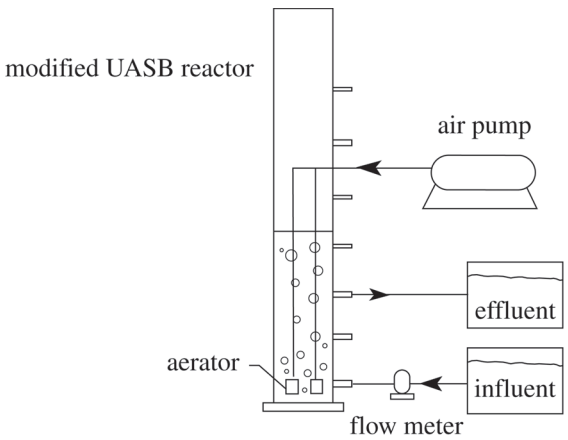

Figure 1. *Configuration of the reactor.*

2.2 The Wastewater for Cultivation

The concentration of influent COD increased from 200–2000 mg/L. The wastewater for cultivation collected from sewage outlet in Dongxing District, Neijiang city. Its COD and NH_4^+-N concentrations were varied in the range of 200–300 mg/L and 4.558–8.670 mg/L, respectively. The COD concentrations in the test were obtained by adding with glucose. To accelerate the formation of aerobic granular sludge, some constant and trace elements were added. The added element include: $CaCl_2$, 30 mg/L; $MgSO_4$, 20 mg/L. Trace element add with 1 ml/L, its component as: $ZnCl_2$, 50 mg/L; $MnSO_4$, 50 mg/L; $AlCl_3$, 50 mg/L; $(NH_4^+\text{-N})Mo_7O_{24}{\cdot}4H_2O$, 50 mg/L; $CoCl_2{\cdot}6H_2O$, 50 mg/L.

2.3 The Landfill Leachate

The landfill leachate was collected from Guibei landfill in Neijiang city. Characteristics of the leachate are listed in Table 1.

2.4 Operation Process

The experiments were conducted at room temperature between 16–24°C. The aeration rate (superficial gas velocity) was 2 cm/s. the reactor cycle consisted of influent, aeration, sediment and settle. The operation cycle time was 4 h; the control parameters of different reactor stage are shown in Table 2. On the condition of influent volume was constant. The choose press was created by enhancing organic loads with the cultivate time increasing, at the same time reducing settle time.

Table 1. Characteristics of the Landfill Leachate.

Parameter	Value
Total COD (mg/L)	44026
NH_4^+-N (mg/L)	3359
Fe (mg/L)	3227
Zn (mg/L)	356
Cd (mg/L)	3.08
Pb (mg/L)	6.70

Table 2. Control Parameters of Various Stages.

Days (d)	1–2	3–4	5–6	7–8	9–10	11–13	14–18
Fill time (min)	5	5	5	5	5	5	5
Aerated time (min)	200	205	210	215	220	223	225
Settle time (min)	30	25	20	15	10	7	5
Draw/idle time (min)	5	5	5	5	5	5	5

This could wash out the small dispersing and aggregate sludge whose settling property was poor, which accelerated the formation and accumulation of granules.

2.5 Fluorescent Staining and CLSM Observation

The hydrated granules were collected from MUASB reactors and stained with fluorescent dyes at different excitation and emission wavelengths (Chen *et al.*, 2007). Particularly, SYTO 63 (20 μM and 100 μl) was added to a sample firstly and shaken on a shaker table for 30 min. Then, 0.1 mol $NaHCO_3$ buffer (100 μl) was added to maintain the solution pH at 9. An FITC solution (10 g/L, 10 μl) was then added, and the solution was immersed for 1 h. Next, a Con A solution (250 mg/L, 100 μl) was added and immersed for another 30 min. Calcofluor white (300 mg/L, 100 μl) was then added for 30 min, and then a Nile Red solution (10 mg/L, 60 μl) was added for another 10 min. After these five staining steps, the sample was washed twice with PBS solution to remove excess stain. Additionally, before observations, a SYTOX Blue solution (2.5 μM, 100 μl) was added to the sample for 10 min. The stained samples were then embedded in paraffin and frozen at –20°C. The 20 μm sections were then cut using a cryomicrotome (Cyrotome E; Thermo Shandon Limited, UK) and mounted onto gelatin-coated (0.1% gelatin and 0.01% chromium potassium sulfate) microscopic slides for CLSM (Leica TCS SP2; Leica, Germany) observations. Samples were examined with a 10× objective and analyzed with Leica confocal software.

2.6 Analytical Methods

COD, NH_4^+-N, mixed liquor suspended solids (MLSS), sludge volume (SV_{30}) and sludge volume index (SVI) were analyzed using methods described in Analytical Methods for the Examination of Water and wastewater (MEP, 2005). pH was analyzed by Leici PHS-25 digital PH meter. The configuration of sludge was examined by optical microscope and its diameter was measured by minimeter. Pb, Cd, Fe and Zn were analyzed with TAS-986 Graphit Furnace Atomic Absorption Spectrophotometry (Beijing Puxi Analysis of General Instrument Co., Ltd., China).

3. RESULTS AND DISCUSSION

3.1 Granulation in MUASB

The MUASB was seeded with flocculating sludge from CASS aeration pool in Neijiang Sewage Treatment Plants. The initial sludge MLSS, SVI and SV_{30} were 5–6 g/L, 33.6 mL/g and 25%, respectively. The seeded activated sludge was deep pitchy colors. The volume of seed sludge added in the reactor was 3.5 L in the beginning of cultivation, and no more sludge was added in the operating process. Figure 2 shows the variations of SV_{30} and SVI during cultivation.

The startup strategy was designed to increase the influent COD gradually and decrease the settle time to promote granule formation while avoiding excess washout of biomass. With the cultivation time increased, the color of sludge changed lighter from pitchy in the first seven days. Granule was visible by the naked eye in the 9th day, and the color of granule sludge in this time was yellowish-brown. The granule sludge appeared and grew gradually with the cultivation time and its settling performance also became better. In the 15th day, the granules were stable, their color changed to yellowish, and their shape were ellipse or irregular globose. Examine under microscopy, it was found that there were many protozoa and metazoa around the

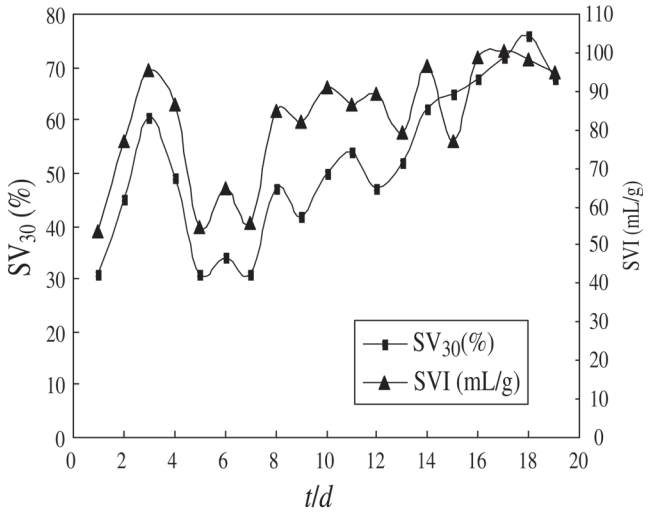

Figure 2. *Variations of SV_{30} and SVI during cultivation.*

granule sludge, indicating high biological activity of granules.

In addition, the results showed that the particle diameter, SV_{30} and SVI of stable granules were 0.2–0.8 mm, 70%, and 95 mL/g, respectively. The highest MLSS reached 8450 mg/L.

3.2 Performance of Leachate Removal in MUASB

On the 19th day, the effluent was changed to mixture of domestic wastewater and landfill leachate with 1 : (13–26) (v/v) and was introduced in five cycles. The control parameters of various react stages were kept the same with 14–18 days cultivation.

3.2.1 Removal of COD and NH_4^+-N in Mixed Wastewater

The average characteristics of influent, effluent mixed wastewater for MUASB during steady state operation with granular sludge (19th–23rd day) are listed in Table 3.The leachate and wastewater were mixed in the ratio of 1 : 18–25. The corresponding profiles of COD and NH_4^+-N are shown in Figures 3 and 4. Although the influent COD increased with cycles, the effluent COD decreased obviously to 98.53 mg/L in cycle 6, which was lower than the standard concentration (1.5 mg/L) according to Standard for Pollution Control on the Landfill Site of Municipal Solid Waste of China (GB 16889-2008). Most of COD was degraded. The COD removal efficiency enhanced with cycles, indicating that granules were domesticated gradually and their function in removing organic material was strengthened. The COD removal efficiency reached 96.21% after 6 cycles. In contrast, conventional UASB reactor had generally a removal efficiency of 50%–70%.(Hu *et al.*, 2002; Osnan *et al.*, 2005; Kennedy *et al.*, 2000). The NH_4^+-N removal quantities per liter increased with ratio of water sample. The lowest residual concentration of NH_4^+-N was 51.4 mg/L in cycle 4, which was still higher than the standard concentration 40 mg/L. These results indicated that only a part of ammonia N entered with the influent and released from organic compounds during fill. During the aeration of 225 min, most of ammonia N was nitrificated and denitrificated in settling time. The uncompleted nitrification could be explained by the COD levels, for nitrifies did not compete well for oxygen with heterotrophy (Wilderer *et al.*, 2001).

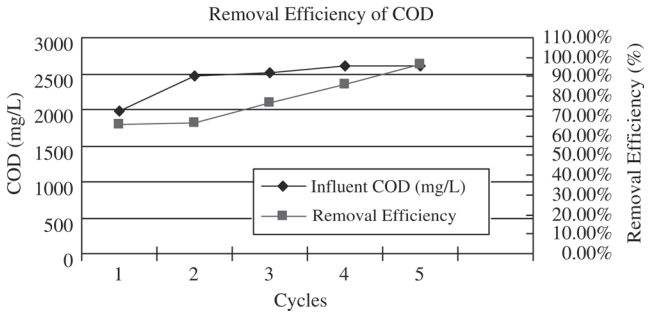

Figure 3. *Cycle profiles of influent COD and its removal efficiency.*

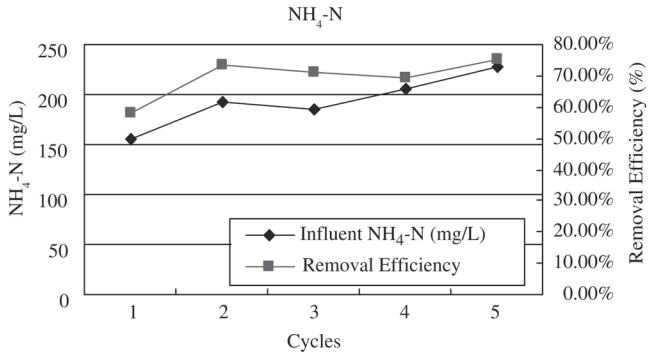

Figure 4. *Cycle profiles of influent NH_4^+-N and its removal efficiency.*

The results from the previous investigations provided supplemental results to support the argument that the variation of temperature (16–24°C) has little or no effect on experimental results. For example, Kishida et al. (2008) and Lemaire et al. (2008) had demonstrated that the temperature of aerobic granular sludge reactor was maintained at 20 ± 2°C and the range of temperature had little effect on their results. Furthermore, de Kreuk et al. (2005) suggested that decreased temperatures from 20–15°C should not be a problem for COD and phosphate removal in a granular sludge system. Therefore, it is reasonable to conclude that such temperature variation (i.e. 16–24±C) had little effect on the results shown in Figures 3 and 4.

3.2.2 The Properties of Aeration Granule Sludge in Treatment of Mixed Wastewater

Table 4 lists some parameters of granule sludge. It

Table 3. The Characters of Influent Mixed Wastewater and Mixing Ratios of Samples in the Test.

Cycle	Influent COD (mg/L)	Influent NH_4^+-N (mg/L)	Ratio of Water Sample
1	11974.96	156.28	4L(1:25)
2	22463.64	191.81	4L(1:20)
3	32520.92	185.81	4L(1:20)
4	42596.82	206.28	4L(1:19)
5	52601.39	228.09	4L(1:18)

Table 4. Some Parameters of Sludge.

Cycle	MLSS (mg/L)	SVI (mg/L)
1	6932	102.42
2	7189	98
3	7325	93
4	7523	492
5	7638	90

can be seen from tale that both the MLSS and SVI increased with cycles. It indicated that the quantity of biomass was increased and settling property of sludge was improved due to the increase of COD removal efficiency with cycles. The decrease of SVI could be explained by granule growth of granule sludge and its augment size.

3.3 Distribution of EPSs in Mature Granules

Figure 5 presents the distribution of EPS in the mature granules cultivated in the MUASB reactor. It could be found that a lot of filamentous bacteria were presented in the fringe region, while cocci were predominant and formed a relatively condense region in the granule interior. Proteins and lipids were distributed throughout the granules, whereas α-polysaccharides were mainly distributed in the core of granules. Few β-polysaccharides and dead cells were detected in the granules. The distributions of total cells and α-polysaccharides were consisted with those of Adav *et al.* (2008). Comparing with Adav *et al.* (2008), we also found that proteins were not only accumulated over the granule interior, but also spread throughout the whole granule.

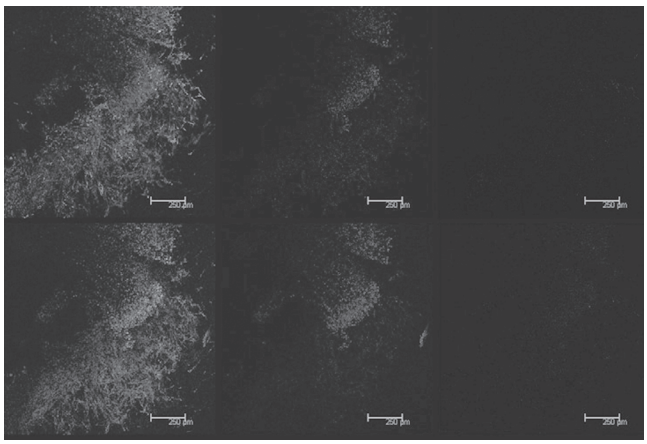

Figure 5. *CLSM images of mature granules with a10× objective lens. (proteins, green; α-polysaccharides, light blue; β-polysaccharides, blue; lipids, yellow; total cells, red; dead cells, violet).*

4. CONCLUSION

On the basis of the results presented and discussed in this study, the following conclusions can be drawn:

1. Granules formed from flocculating sludge in MUASB within 9 days. The granules had an average diameter of 0.5 mm during steady state operation, and showed much better settling properties than the flocculating seed sludge.
2. Granules cultivated in the MUASB were capable of removing COD, NH_4^+-N from landfill leachate. This is the first study to demonstrate that aerobic granules can effectively treat landfill leachate mixed with wastewater.
3. As for the mature granules, CLSM observation revealed that a lot of filamentous bacteria were presented in the fringe region, while cocci were predominant and formed a relatively condense region in the granule interior. Proteins and lipids were distributed throughout the granules, whereas α-polysaccharides were mainly distributed in the core of granules. Few β-polysaccharides and dead cells were detected in the granules.

ACKNOWLEDGEMENT

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Antifungal Activity of the Essential Oils of *Pyrethrum leptophyllum* Stev. ex Bieb.

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ABSTRACT: Chemical composition of essential oil extracted from *Pyrethrum leptophyllum* Stev. ex Bieb., a plant of Caucasian endemic flora, was examined. Essential oil was extracted using a hydrodistillation method and above-ground portions of the plant (0.1%) during its blooming season. According to chromatospectrometer results, major compounds of essential oil are α-Thujone (85.004%) and β-Thujone (10.566%). It has been determined that water extract and essential oil from Pyrethrum leptophyllum is responsible for antifungal activity against *Trichoderma lignorum*, *Fusarium oxysporum*, and *Aspergillus niger*.

INTRODUCTION

NOT only do synthetic preparations exist among antimicrobial medicines but they also exist in herbal preparations that have low and high antimicrobial activity levels. Importance of these herbal preparations is significantly increasing. A number of studies [4–6, 8, 10–11, and 13–16] have been performed on antimicrobial characteristics of various plant species (e.g., *Myrica gale*, *Acorus calamus*, *Euphorbia amygdaloides*, *Achillea biebersteinii*, *Teucrium hircanicum*, *Ferula latisecta*, *Mozaffania insignis*, *Daucus carota*, *Tanacetum cadmium*, *Salvia desoleana*, and others) and also on their bioactive materials like essential oils, extractions, saponins, flavonoids, and more. However, there have not been enough studies carried out on other *Pyrethrum* species.

This plant was used in this study as the research object of interest. There are more than 100 genera in the world's flora, which is widely distributed in Europe, North Africa, and in ex-Soviet areas. While 44 species of *Pyrethrum* Zinn occur in Caucasia (i.e., Dagestan and North Caucasia), 16 to 24 of its species may be found in Azerbaijan [1, 2, and 9]. Aforementioned species contain bioactive materials like essential oils, sesquiterpene lactones, coumarin, flavonoids, vitamin C, fenolcarbons, organic acids, and more. Long ago some *Pyrethrum* species were used as an agent for medication for diarrhoea, worms, wound-healing, antipyretic, and tonicity. It was also utilized for treatments of Siberian ulcer, cancer, fracture, and nervous and skin disease. *Pyrethrum carneum*, a member of *Pyrethrum* genus, is still being used in medicine as an antacid agent covering the upper part of stomach mucous. Antibacterial, Protistocid, and Fitoncid activities of the following plants were investigated: *Pyrethrum balsamita*, *P. coccineum*, *P. parthenifolium*, *P. punctatum*, *P. sevanense*, *P. fruticulosum*, and *P. sericeus* [12].

There is little to no information available in the literature regarding *Pyrethrum leptophyllum*. Therefore, essential oil and water extractant from the Azerbaijani *Pyrethrum leptophyllum* plant and its essential oil's antifungal activity was analyzed in order to increase number of antifungal agents.

MATERIALS AND METHODS

Regarding plant material, above-ground portions of the *Pyrethrum leptophyllum* plant were obtained from a

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valley's rocky slopes 1,448 m above sea level and located near the Qırız Dehne Village in the Guba District of Azerbaijan on August 20, 2008. During sampling, a GPS displayed coordinates as 41° 13′ 29.5″ N and 048° 17′ 31.6″ E. Voucher specimens were deposited at the Herbarium of the Sciences Institute of Botany (BAK) in Baku, Azerbaijan. Aerial parts of Pyrethrum leptophyllum were subjected to hydrodistillation [3] for isolation of essential oils.

Gas Chromatography (GC) was conducted using a Shimadzu GC-17A with a capillary CP-SIL 8 CB column (15 m × 0.25 – 0.39 nm). Nitrogen was used as the carrier gas at a constant flow rate of 5 ml/min. Oven temperature was held at 60°C for 2 minutes, then programmed to 250°C at a rate of 6°C/min for 5 minutes, and then held to 280°C at 15°C/min for 6 minutes. The injector and detector (FID) temperatures were kept at 280°C and 300°C, respectively.

Gas Chromatography/Mass Spectroscopy (GC/MS) analysis was carried out using an Agilent 6890N Network GC system combined with an Agilent 5975 inert Mass Selective Detector. GC setup used a capillary column Agilent 19091S-433 HP-5MS 5% Phenyl Methyl Siloxane. Specifications were set for nominal length at 30.0 m, nominal diameter at 250.00 μm, and nominal film thickness at 0.25 μm. Oven temperature was held at 70°C for 2 minutes, then programmed to 280°C at a rate of 5°C/min, and then held for 6 minutes. Helium was used as a carrier gas with nominal unit pressure at 7.64 psi, average velocity at 36 cm/sec, initial flow at 1.0 mL/min, split ratio at 40:1, and injected volume at 0.50 μL. Components' identification was confirmed by comparison to a mass spectral datum using Wiley and Nist electronic libraries. Percentages for components were calculated from GC peak areas using the normalization method.

Trichoderma lignorum, *Fusarium oxysporum* and *Aspergillus niger* were used as fungi all supplied from the AMEA Institute of Microbiology. Antifungal activity was measured in three steps as follows: (1) development of fungus in solid media, (2) development of fungus in liquid media, and (3) development of fungus in Czapek's media by adding essential oil extracted from *Pyrethrum leptophyllum*. The most common [7] methods were used on cultivating fungus. Material extracted from the thin-leafed *Pyrethrum leptophyllum* plant was crushed into small pieces ranging from 0.5 to 1 cm in size and wetted with 55–60% water. Therefore, fungi can develop in solid media. The pH level was relatively constant ranging from 6.5 to 7. The substrate, extensively washed with culture, was laid into Petri dishes and sterilized at 1 atm of pressure for 45 minutes. After sterilization, fungal biomass was equally inoculated into Petri dishes and incubated at 25–27°C. Colony counts were performed on each of the dishes at 3, 5 and 7 days following inoculation.

DISCUSSION

Pyrethrum leptophyllum refers to several old world plants from the genus Asteraceae which grow to a height somewhere between 45 cm and 60 cm. They are commonly known as a Caucasian endemic plant. They are xerophytes and hemicriptofit. Also, they are known to flourish at altitudes above 2,500 m in mountain scrubs, rocky slobs, waste places, and around rocky cliffs. Flowering period occurs in June and July in the Northern Hemisphere and it spreads its seed between August and September. It is widely distributed around big Caucasian heights of Azerbaijan. It can easily be found in the forest and mountainous terrain of the Guba province (5).

The Amount of essential oil extracted from the above-ground portion of *Pyrethrum leptophyllum* is 0.1%. Five elements have been identified related to chemical composition of the essential oil. They are displayed in Table 1. Maximum percent for Oxygen Monoterpen is 99.108%, α-Thujone is 85.004%, and β-Thujone is 10.566%. Minimum percent for nitrogen compounds inside essential oil is 0.892% (see Table 1).

Appropriate methods as previously mentioned in the Materials and Methods section and displayed in Figure 1 were implemented for monitoring development periods for fungi in solid media. *Trichoderma lignorum* colonies and *Aspergillus niger* colonies had a similar growing speed ranging from 3 days to 5 days. Day 7, *Trichoderma lignorum* colonies' growing speed significantly exceeded *Aspergillus niger* colonies' growing speed. Over the 7 day period, development of these three types of fungi were on average 9, 7, and 8 cms.

***Table 1. Chemical Composition of the Essential Oil from* Pyrethrum leptophyllum.**

Compound	t, minutes	w, %
1,8 –Cineole	7,340	1.501
α-Thujone	8,208	85.004
β-Thujone	8,309	10.566
4-Terpineol	8,956	2.037
Cyclopropanecarbohydroxamin acid, 2,2-Dimethyl-3 (2-methyl-1-) propenyl	9,345	0.892

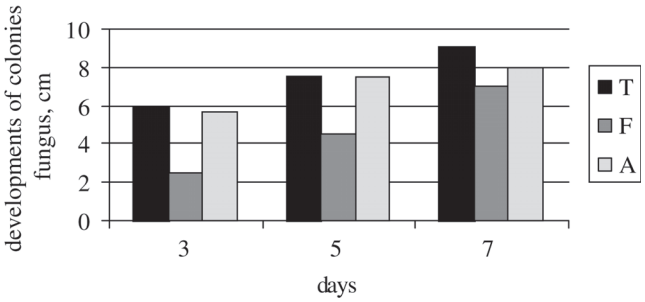

Figure 1. *Development processes for* Trichoderma lignorum *(T), Fusarium oxysporum (F), and* Aspergillus niger *(A) colonies in solid media prepared with* Pyrethrum leptophyllum.

Trichoderma lignorum colonies grew 1.6 cm in 5 days and 3.1 cm in 7 days. *Fusarium oxysporum* colonies grew 2 cm in 5 days and 4.5 cm in 7 days. *Aspergillus niger* colonies grew 1.8 cm in 5 days and 2.3 cm in 7 days. Thus, investigations suggest that *Pyrethrum leptophyllum* plants are a good nutritious substrate for tested fungi cultures.

Results suggested nectar extracted from *Pyrethrum leptophyllum* was favourable for development of these fungi types. Samples taken to define antifungal activity were collected from the upper part of the plant and were extracted by heating with a 1:10 water wash. Then, extraction was cooled, filtered, and poured into 100 ml glass bottles. It was sterilized at 0.5 atm pressure for 45 minutes. The test was conducted at pH levels of 6, 5, and 7. Fungi strains were inoculated into an aqueous extract inside a test tube. Then, they were incubated at 25–27°C for seven days.

Czapek's media was used for comparing results. After filtration of a liquid culture, weight of fungal biomass was measured. Then, it was dried at 98°C until it reached a constant weight. Results are as displayed in Figure 2.

As seen in Figure 2, it has been determined that aqueous extract from the *Pyrethrum leptophyllum* plant has strong fungistatic activity in comparison to control tests. The aqueous extract displayed a more inhibitory effect against *Trichoderma lignorum* at 0.29 g/l dry weight and a less inhibitory effect against *Aspergillus niger* at 0.84 g/l dry weight.

Czapek's media was prepared to examine the antifungal activity of essential oil from the *Pyrethrum leptophyllum* plant. It was sterilized in an autoclave for 45 minutes at 0.5 atm of pressure. Next, an alcoholic solution of essential oil was poured into test tubes at 0.1%, 0.3% and 0.5%, respectively. However, no essential oil was poured into control test tubes. Biomasses of

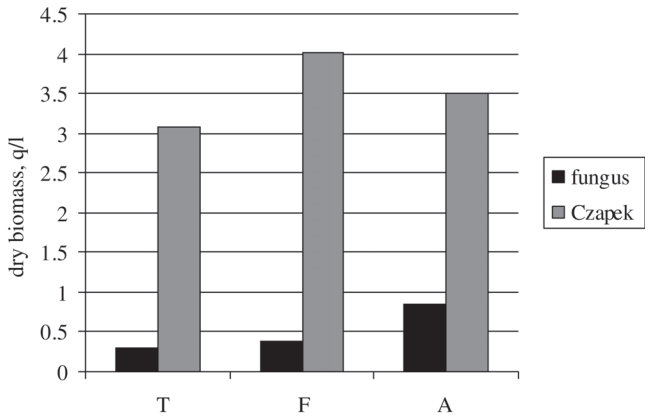

Figure 2. *Effects of aqueous extracts from* Pyrethrum leptophyllum *on Trichoderma lignorum (T),* Fusarium oxysporum *(F), and* Aspergillus niger *(A) fungal colonies.*

Trichoderma lignorum, *Fusarium oxysporum*, and *Aspergillus niger* fungal cultures were inseminated inside test tubes in the same fashion. Thereafter, test tubes were incubated for seven days at 25–27°C. After day seven, fungal inoculants were filtered, dried, and weighed.

Fungistatic activity of essential oil was evaluated by physically weakening fungal cultures' development. Results are as displayed in Figure 3. Results also demonstrated all essential oil concentrations of *Pyrethrum leptophyllum* had a negative impact on *Aspergillus niger* fungal cultures and eventually killed them. Concentration at 0.5% essential oil had the highest inhibitory activity against fungal cultures prepared by

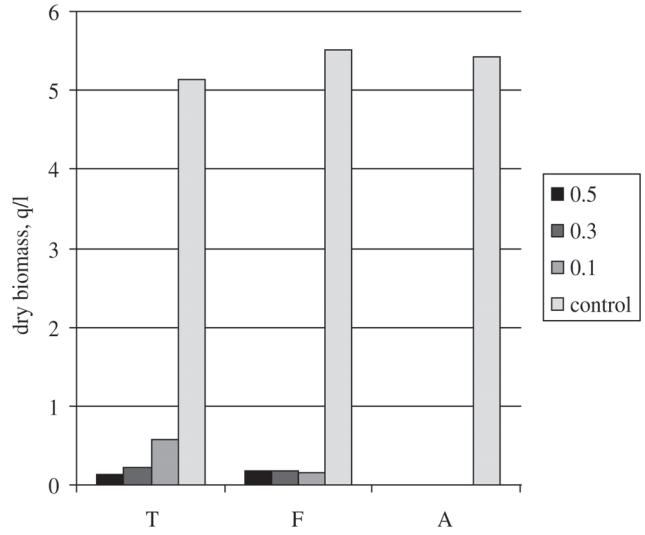

Figure 2. *Effects of different essential oil concentrations of* Pyrethrum leptophyllum *on* Trichoderma lignorum *(T),* Fusarium oxysporum *(F), and* Aspergillus niger *(A) fungal colonies' development.*

Trichoderma lignorum strains. Concentration at 0.1% essential oil had the lowest inhibitory activity against fungal cultures. Their dry weights became 0.14 g/l and 0.58 g/l, respectively. Regarding fungal cultures prepared by the *Fusarium oxysporum* strain, all essential oil concentrations suggested similar fungistatic activities as it did for *Trichoderma lignorum*. Relevant dry weights were reported as 0.18 g/l, 0.17 g/l, and 0.16 g/l, respectively.

CONCLUSION

Essential oil (0.1%) was extracted from above-ground portions of *Pyrethrum leptophyllum* plant. Five compounds were identified in essential oil extracted from above-ground portions of the *Pyrethrum leptophyllum* plant. Major compounds were determined to be α- Thujone at 85.004% and β-Thujone at 10.566%. It was determined that essential oil and water extracted from *Pyrethrum leptophyllum* was fungistatic and provides a fungicide against *Trichoderma lignorum*, *Fusarium oxysporum*, and *Aspergillus niger* fungi cultures. Its nectar was beneficial for fungal development. Essential oil extract has more antifungal potential than water extracted from the inherent plant. Either water extracted or essential oil extract can be used as an antifungal material.

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Table 5. Comparison of state-of-the-art matrix resins with VPSP/BMI copolymers.

Resin System	Core Temp. (DSC peak)	T_E	Char Yield, %
Epoxy (MY720)	235	250	30
Bismaleimide (H795)	282	>400	48
VPSP/Bismaleimide copolymer			
C379: H795 = 1.9	245	>400	50
C379: H795 = 1.4	285	>400	53

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