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DÉGRADATION AÉROBIE DES BIOSOLIDES MUNICIPAUX ET RÉCUPÉRATION DE BIOCATALYSEURS POUR LE TRAITEMENT DES EAUX USÉES

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RÉSUMÉ

Le volume élevé de biosolides municipaux et industriels (BS) produits dans le monde entier est une source de préoccupation majeure pour les écologistes. Actuellement, la production mondiale de BS est estimée à environ 100 à 125 millions de tonnes par an et devrait augmenter continuellement. Au Canada, 6,6 millions de tonnes de BS stabilisés secs sont produites chaque année par plus de 3 500 stations d'épuration des eaux usées (STEP). La gestion et l'élimination des BS sont très préoccupantes pour de nombreuses municipalités, car cela peut représenter plus de la moitié du coût total du traitement des eaux usées. La valorisation des BS pour la production de biogaz présente un grand avantage. En effet, la digestion anaérobie des BS peut être utilisée pour produire du biogaz comme combustible si celui-ci est correctement collecté et purifié. Outre cet avantage, les BS représentent une menace environnementale à travers la production de gaz à effet de serre (GES), et la production de lixiviat contenant de nombreux contaminants organiques (incluant des contaminants organiques à l'état de traces (TrOCs), tels que les composés pharmaceutiques actifs (PhACs) et pesticides). Ainsi, à partir des sites d'enfouissement et de l'amendement des sols avec les BS municipales, ces contaminants peuvent se retrouver dans les eaux souterraines. Pour remédier à cela, le Québec a mis en place des politiques strictes en matière d'élimination des BS. On compte parmi les mesures prises l'interdiction du déversement des BS dans les décharges et établit une taxe verte qui est prélevée en fonction de l'inflation pour chaque tonne de BS incinérée ou mise en décharge au Québec.

Cependant, avec l'aide de technologies de bioprocédés avancées, ces BS peuvent être traités et utilisés simultanément comme matière première pour la production de produits de grande valeur ou de précurseurs de produits de grande valeur. Ce travail de recherche fournit une évaluation des principales méthodes d'élimination (réduction du volume et des contaminants) et passe en revue l'état des processus biotechnologiques, en particulier pour la digestion aérobie pour la production d'enzymes hydrolytiques et lignolytiques à partir des BS.

En premier lieu, l'impact des microorganismes endogènes a été étudié avec ou sans co-traitement. En ce sens, divers traitements comprenant l'utilisation de microorganismes indigènes présents dans les BS, l'effet combiné d'un prétraitement enzymatique, la biostimulation par l'ajout d'une source externe de carbone et l'effet synergique de la biostimulation et du

prétraitement enzymatique ont été étudiés pendant 28 jours. En raison de diverses stratégies de traitement, une réduction totale des solides en suspension de 12% à 23%, une élimination des PhACs de 44% à 62% et une élimination des pesticides d'environ 10% à 54% ont été observées. Aussi, pour améliorer l'élimination des solides en suspension des PhACs et pesticides, l'effet de quatre stratégies de prétraitement différentes (ultrasonication, congélation-décongélation, addition enzymatique et traitement alcalin), suivis d'une bioaugmentation avec la souche de bactérie *Bacillus subtilis* a été étudiée. Après 28 jours de traitement, la bioaugmentation a amélioré l'élimination totale des solides en suspension par 8 à 54% et une l'élimination des PhACs par 21 à 80% et une l'élimination des pesticides par 22 à 76%. La production d'un cocktail d'enzymes extracellulaires (ex. : laccase, peroxydase, glucose oxydase, lipase, phosphatase, estérase…etc.) à la suite de la bioaugmentation des BS avec *B. subtilis* a été observée. Ces enzymes peuvent être impliquées dans l'élimination des contaminants présents dans les BS ainsi que dans la réduction des solides observée.

Cette étude est une approche bio-intégrée, où les enzymes produites lors de la digestion aérobie des BS ont été évaluées pour l'élimination des contaminants organiques présents dans les BS issus du traitement des eaux usées municipales. Pour souligner cela, la bioaugmentation des BS avec la souche de champignon *Aspergillus niger* a été évaluée dans le but de produire une enzyme d'intérêt telle que la glucose oxydase (GOD). La GOD sécrétée par *A. niger*, a été utilisée pour le processus d'oxydation avancé basé sur le bio-Fenton pour la dégradation de 15 PhACs d'intérêt dans l'eau. En somme, l'utilisation des BS comme substrat par les microorganismes étudiés a permis d'une part de diminuer les contaminants organiques et, d'autre part, de réduire le volume des BS.

Mots clés : Biosolides; Produits à valeur ajoutée; Contaminants; Digestion aérobie

Abstract

The high volume of municipal and industrial biosolids (BS) produced all over the world is a cause of major concern to the environmentalists these days. Currently, the worldwide production of BS is estimated to be around 100-125 million tonnes per year and is expected to increase continuously. According to Canadian context reported on, 6.6 million tonnes of dry stabilized BS are generated yearly by more than 3500 wastewater treatment plants (WWTPs). Management of BS is primarily handled as a technical matter. The disposal of BS is of great concern for many WWT facilities because it accounts for over half of the total cost of wastewater treatment. BS disposal in landfill sites leads to a great advantage of biogas generation by anaerobic digestion which can be used as a fuel if the biogas is properly collected. Apart from adding advantages, it poses environmental threat such as trace organic contaminants (TrOCs), green house gases (GHGs) production, potential contaminant leachate into the ground water system at the landfill site as well as soil amendment of municipal BS . Stringent laws in BS disposal are followed in Quebec which focuses on restraining from the dumping of BS in landfills and green tax is levied based on the inflation for each ton of biosolid that is incinerated or landfilled in Quebec.

However, with the help of advance bioprocess technologies these biosolids can be treated and simultaneously used as a raw material for the production of high value products or high value product precursors. This current research work delivers an assessment of the leading disposal methods (volume and contaminant reduction) and reviews the state of biotechnological processes, particular to aerobic digestion for the production of hydrolytic and lignolytic enzymes from BS.

During aerobic digestion, various pretreatments including utilization of indigenous microbes present in BS, the effect of an enzymatic pretreatment, biostimulation by the addition of an external carbon source and the synergic effect of biostimulation and enzymatic pretreatment were studied for 28 days. As a result of various treatment strategies, the total suspended solids reduction of 12-23% ,total PhACs removal of 44-62% and total pesticides removal around 10-54% were observed. Further, to enhance the total suspended solids, total PhACs and total pesticides removal, effect of four different pre-treatment strategies (ultrasonication, freeze-thawing, enzymatic and alkaline addition) subsequent bioaugmentation with *Bacillus subtilis* for

28 days were studied. Impact of bioaugmentation improved the total suspended solids removal by 8-54%, total PhACs removal by 21-80% and total pesticides removal by 22-76%. The production of enzymatic cocktail by bioaugmented along with indigenous microorganisms, which contain high laccase, peroxidase, glucose oxidase, lipase, phosphatase, esterase activities and other activities related to elimination of contaminants present in the BS. This study is bio-integrated approach, where the enzymes producing during aerobic digestion of BS is evaluated to reduce the contaminants present in water or wastewater. To emphasize that, bioaugmentation of *Aspergillus niger* to BS to produce glucose oxidase (GOD), which was utilized for bio-Fenton based advanced oxidation process for the partial removal of 15 pharmaceutically active compounds in water. Therefore, the use of BS residuals as a substrate can decrease the contaminants on the one hand and, on the other hand, it can reduce the volume of BS.

Keywords: Biosolids; Value-added products; Contaminants; Aerobic digestion

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LISTES DES ABRÉVIATIONS

BS – Biosolids

BTEX- Benzene, toluene, ethylbenzene and xylene

CAGR- Compound annual growth rate

COD - Chemical Oxygen Demand

DD- Degree of disintegration

DHA- Dehydrogenase

DNS- Dinitro salicylic acid

DOSS- Dioctyl Sodium Sulfosuccinate

EDTA- Ethylenediaminetetraacetic acid

EMP- Embden-Meyerhof-Parnas

EPS - Extracellular polymeric substance

ESI-Electro spray ionization

FDA- Fluorescein diacetate

GHG- Green house gases

GOD- Glucose Oxidase

GYM- Glucose, yeast extract and malt extract

MTBE- Methyl tert-butyl ether

NFRV- Nitrogen fertilizer replacement value

OM - Organic Matter

PAHs- Polycyclic aromatic hydrocarbons

PCBs- Polychlorinated biphenyls

PhACs- Pharmaceutically activated components

PHAs- Poly hydroxy alkanoates

PSB- Phosphate solubilizing bacteria

PT - Pretreatment

RSM- Response surface methodology

SCOD - Soluble Chemical Oxygen Demand

SS- Suspended solids

TDS - Total Dissolved Solids

TEQ – Toxic equivalent quotient

TOC- Total Organic Carbon

TrOCs - Trace Organic Contaminants

TS - Total Solids

TSS - Total Suspended Solids

UPLC-MS - Ultra performance liquid chromatography - mass spectrometry

VAP- Value added products

VFA-Volatile fatty acids

VSS - Volatile suspended solids

WWE - Wastewater Effluent

WWTP - Wastewater Treatment Plant

Chapitre 1
INTRODUCTION GÉNÉRALE

Biosolids are the treated continuously-produced solid by-product of a wastewater treatment plant (WWTP). The high volume of municipal and industrial biosolids produced all over the world is a cause of major concern to the environmentalists these days. In the current scenario of increasing global population, the generation of solid wastes like BS is bound to increase remarkably [1]. According to Canadian context reported on, 660,000 metric tons of dry biosolids are generated yearly by more than 3500 WWTPs [2]. Disposal of BS were carried out by the processes such as incineration, land filling, ocean dumping, in which the last two methods of disposal mentioned above, have a serious disadvantage of causing land and water pollution, respectively. Currently, the costs associated with the disposal of BS represent approximately 50% of total costs of operation of municipal WWTPs [3,25]. The improper disposal of solid wastes like biosolids pose a serious threat to the environmental quality leading to problems like surface and groundwater contamination, degradation of land, and food chain contamination etc. Due to the high organic matter and nutrient content of biosolids, there are many alternative choices for their disposal besides being landfilled or soil application.

According to Québec *Sustainable Development Act* context : “When there are threats of serious or irreversible damage, lack of full scientific certainty must not be used as a reason for postponing the adoption of effective measures to prevent environmental degradation”[4]. Climate change is the serious and irreversible threat to the environment and human health, where most of the government believes release of green house gases (GHGs) to the environment is the major cause of climate change. This contribution of GHG production to the environment increases the probability of increase in the rate of global warming. GHG production, potential contaminant leachate into the ground water system at the landfill site hinders the possibility of disposal of BS in landfills. Stringent laws in BS disposal are followed in Canada which focuses on restraining from the dumping of BS in landfills and green tax (up to $30/ton-$100/ton) is levied based on the inflation for each ton of biosolid that is incinerated or landfilled in Quebec.

Quebec Government, which in its "Québec Policy on Climate Change Action Plan and its Residual Materials Management", initiate the promotion of the recycling of urban organic waste, including the use of treated sludges (biosolids) and also it ensures the complete ban of organic material which include municipal biosolids from landfills or incineration by the year 2020. Claude Villeneuve reported that, if all of Québec's municipal biosolids were recycled as fertilizer, urban emissions would plummet by some 500,000 tons CO_2 equivalent a year [3]. Canada has led to extensive research on the valorization and volume minimization of biosolids. However, only 51% of the total biosolids (38% as soil amendment and 13% as compost) in Quebec are actually utilized for valorization [3].

Owing to the high content of carbon, nitrogen, phosphorus, potassium, and other nutrients, the use of biosolids from municipal wastewater treatment plants as a major substrate for production of enzymes represent a promising alternative for sludge management. However, the constituents of biosolids are characterized by the presence of certain toxic trace metals and emerging trace organic contaminants that is a global environmental concern when it comes to their land application. Heavy metal concentration in biosolids produced in different countries may differ due to dissimilar WWT technologies adopted or because of variation in generated wastewater chemical composition. In addition, several other trace organic contaminants such as pesticides, insecticides, disinfectants, pharmaceuticals, detergents, personal care products, steroid hormones and various other inorganic salts are present in wastewater and finally in the processed biosolids. The presence of these contaminants and pathogens can cause problems with the quality of the environment and the health human.

The growing recognition of biosolids as an untapped resource demands for improved WWTPs, which will reduce the solid volume and the contaminants content while increasing the recovery of useful by-products that support the transition to a circular economy [5].

1.1 NOVELTY OF THE PRESENT STUDY

Globally the biosolids that are produced from WWTPs are increasing exponentially. The disposal of biosolids is of great concern for many WWT facilities because it accounts for over half of the total cost of wastewater treatment. Due to stringent regulatory norms, the wastewater treatment plants (WWTPs) face confrontations and challenges in adapting a technology for the effective management of biosolids. However, with the help of advanced bioprocess technologies these biosolids can be treated and simultaneously used as a raw material for the production of high value products or high value product precursors.

This thesis aims in directing an integrated bio approach towards utilizing biosolids via microbial treatment to cater biosolids disposal by reducing their volume, simultaneously by reducing organic contaminants present in it. Biosolids is a potential source of microorganisms and mineral nutrients which also includes pathogens and organic contaminants. Addition of oxidative and hydrolytic enzyme producing bacteria/fungi into the biosolids could result in improved production of enzyme cocktails compared to that observed in inducible compounds in the media thus reducing the use of chemicals. As the enzymatic cocktail obtained from the biosolids is a combination of lignocellulosic and hydrolytic enzymes which have the potential for removing the trace organic contaminants and organic matter. Therefore, the use of biosolid residuals as a substrate can decrease the contaminants on the one hand and, on the other hand, it can reduce the biosolids volume.

This research work provides a useful approach towards utilization of the abundantly produced biosolids to value added products like enzymes. The proposed integrated approach will thus meet the growing concerns of Canadians regarding the presence of these contaminants in the aquatic environment. Thus, the synergistic application of microbially treated biosolids over volume reduction and contaminants removal will provide an efficient, easily applicable, environmentally friendly solution to the prevailing environment issues. This research work will lay a platform for using the otherwise repudiated biosolids to derive the widely applicable enzymes of high market value within the realm of the principles of sustainable development.

1.2 OBJECTIVES OF THE STUDY

The overall objective of this thesis is about development of an innovative integrated bio approach allowing the reduction and treatment of biosolids while producing enzymes that can be valued for the treatment of contaminants of emerging concern in water and in other applications, so that the research approach will produce low-cost biocatalysts of industrial interest and achieve true, long-term, sustainability.

To meet this general objective, we have divided these into specific objectives

- Testing the potential of indigenous microorganisms with /without biostimulation on biosolids for their volume reduction and concomitant contaminants removal.
- Treat biosolids with bacteria/fungi to evaluate their performance on production of enzyme cocktail, volume reduction and contaminant removal.
- Exploration and validation of oxidative enzyme mediated bioprocess for the removal of emerging contaminants from water and to evaluate the robustness and reliability of environment biotechnologies.

1.3 STRUCTURE OF THE THESIS

Chapter 1 will present the general introduction of the current study. Chapter 2 will consist of a review of literature to the current research work, emphasizing on the significance of biotechnological approach for the conversion to organic waste biomass into value added resources. This chapter also delivers an assessment about three major parameters such as contaminant removal, volume reduction and value-added products (VAP) production from aerobically digested municipal wastewater biosolids. This chapter will also address the importance of pretreatment (PT) over aforementioned parameters in aerobic digestion of biosolids. Further, this also features research gaps of the present study and highlight the purposes of the study.

Chapter 3 presents the methods supporting the research work. Chapter 4 will give a brief introduction about physio-chemical characterization of bioslurry (mixture of wastewater and biosolids) which includes solid content, contaminants status and enzyme profile obtained from

local municipal WWTPs. And also comparing various treatment approaches such as utilization of indigenous microbes present in bioslurry (T1), enzymatic PT (T2), biostimulation by the addition of an external carbon source (T3) and the synergic effect of biostimulation and enzymatic PT (T4) in bioslurry to evaluate their efficiency on solids reduction and contaminants removal. This chapter also delivers the changing aspect of enzyme profile produced by indigenous microorganisms during the enhanced bioremediation process of biosolids.

Chapter 5 first focussed on introducing four different PT techniques to BS such as ultrasonication, freeze-drying, alkaline addition and enzymatic PT for the disintegration of sludge which also includes the TrOCs removal and solid reduction. Further disintegrated biosolids undergo *Bacillus subtilis* aided aerobic digestion for 28 days during the course of the experiment various parameter such as chemical oxygen demand (COD), enzyme cocktail, solid reduction and TrOCs removal were also investigated.

Chapter 6 will discuss about the utilization of municipal BS for the production of glucose oxidase (GOD) by augmenting fungal strain *Aspergillus niger*. Optimization of various parameters such as BS concentration, pH, inoculum age, type and size were studies to maximize the production of GOD. Further produced enzyme was used to create the bio-fenton process to remove pharmaceutically active compounds (PhACs) from aqueous solution.

Chapter 7 will include the summary and conclusions of the present study.

Chapter 2, 4, 5 and 6 were articles and already published in peer reviewed journals.

CHAPITRE 2

VALORISATION DES BIOSOLIDES ET PRODUCTION CONCOMITANTE DE PRODUITS À VALEUR AJOUTÉE : UNE REVUE DE LA LITTÉRATURE

Biosolids valorization and the concomitant production of value-added products: A literature review

Avant- propos

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Résumé français : Les biosolides (BS) représentent la matière sèche organique produite par les stations d'épuration des eaux usées (STEP) et il y a une augmentation linéaire de la production mondiale. Actuellement, la production mondiale de biosolides est estimée à environ 100 à 125 millions de tonnes sèches. D'ici 2025, on estime que la production annuelle sera d'environ 150 à 200 millions de tonnes. Les industries de traitement des eaux usées du monde entier s'efforcent donc de créer une base de gestion et de valorisation durable des biosolides municipaux, car ces dernières sont riches en nutriments, en matières organiques et contiennent des contaminants. La gestion des biosolides à l'aide de technologies de valorisation respectueuses de l'environnement est un défi global majeur. De nos jours, le traitement des biosolides par des processus biologiques et la contribution à l'économie du processus met l'accent sur la valorisation des biosolides comme une ressource pour la production de produits à valeur ajoutée (VAP).

Cet article présente une évaluation des principales méthodes de traitement (sur la base de la réduction du volume et des contaminants) et passe en revue l'état des processus biotechnologiques pour la récupération des VAP des biosolides municipaux. Une revue des bioprocédés, en particulier des procédés de digestion aérobie, est présentée pour donner un aperçu holistique de ce domaine de recherche en pleine croissance. Cette étude met également en lumière les approches de réduction des polluants et de récupération de ressources telles que les enzymes, les bioflocculants, les bioplastiques et les biopesticides comme moyen de représenter les biosolides comme une opportunité potentielle pour la production de VAP. En raison du potentiel et de la rentabilité du processus de récupération des VAP, seules quelques technologies ont été mises en œuvre et le passage des STEP aux installations de récupération des ressources présente dans les BS à grande échelle est encore loin d'être une réalité.

Mots clés : Biosolides; Produits à valeur ajoutée; Contaminants; Digestion aérobie

Abstract: Biosolids (BS) are organic dry matter produced from wastewater treatment plants (WWTPs). The current yearly worldwide production of BS is estimated to be around 100-125 million tonnes and is expected to continuously increase to around 150-200 million tonnes by 2025. Wastewater treatment industries across the globe strive to achieve a green and sustainable manufacturing base for the management of enormous amounts of municipal BS, which are rich in nutrients and organic dry matter along with contaminants. The management of these organic-

rich wastes through environment friendly recovery technologies is a major challenge. Alleviation of waste biomass disposal by biological development processes and contribution to the economics of the process lays the emphasis on the transformation of waste resources into value-added products (VAP). This article delivers an assessment of the leading disposal methods (based on volume and contaminant reduction) and reviews the state of biotechnological processes for VAP recovery from municipal wastewater sludge (untreated solid waste residual) and biosolids (stabilised solid waste which meets criteria for their use in land). A review on the aerobic digestion processes is presented to provide a holistic overview of this growing research field. Furthermore, the article also sheds light on the pollutant reduction and resource recovery approaches for enzymes, bioflocculant, bioplastics, biopesticides, and biogas as a mean to represent biosolids as a potential opportunity for WTPs. Due to potential and cost effectiveness of VAP resource recovery process, only a few technologies have been implemented and a shift from WTPs towards waste resource recovery facilities is still far from being achieved.

Keywords: Biosolids; Value-added products; Contaminants; Aerobic digestion

2.1 INTRODUCTION

Biosolids (BS) are nutrient-rich organic dry matter produced from wastewater treatment plants (WWTPs). Apart from their nutrient content, BS are also rich in microbial sources, pathogens, organic and inorganic contaminants [6,7]. There are many published works that describe the production of these solid wastes around the globe [8–13]. The amount of BS produced annually worldwide has increased dramatically due to the construction of treatment plants and upgrading of existing facilities as a result of increased water demand and WWTP regulations [14]. Currently, the worldwide production of BS is estimated to be around 100-125 million tonnes and is expected to continuously increase to around 150-200 million tonnes by 2025 [1]. The high volume of municipal and industrial BS produced all over the world is a major cause of concern to environmentalists these days. At the current global population growth rate, the generation of solid wastes like BS is bound to increase dramatically. In Canada, for instance, 3500 WWTPs produce around 660,000 metric tons of dry BS yearly [15,16]. These BS are either disposed of or used in land applications [15]. To promote the beneficial use of the otherwise discarded BS, the Canadian Council of Ministers of Environment (CCME) has reviewed and published the

accepted methods of BS treatment. According to this report, dewatering, drying in rotary vacuum dryers and nutrient recovery from wastewater is employed to improve the quality of BS produced [15].

The enormous quantity of BS produced from WWTPs always poses difficulties for environmental scientists and engineers due to the handling and disposal processes and thus, its management requires careful consideration [17]. The presence of contaminants and pathogens in BS, which are hazardous to human and animal health, makes government bodies rethink their use in agricultural land application. Alongside the waning of traditional BS disposal routes, due to mounting pressure from the public, there is a great demand for environmentally acceptable and cost effective alternative routes [18].

Generation of these solid wastes is increasing due to rapid global urbanization and has created a threat to the environment, forcing public/private solid waste generators to rethink current BS management strategies [19]. Furthermore, an increasing demand of primary energy due to the depletion of fossil fuel reserves along with the combination of various scenarios such as climate change, public awareness and recent advancements in technology have driven the attention towards renewable energy [19]. Moreover, from an industrial point of view, manufacturing of commercial microbial-based value-added products (VAP) requires an inexpensive route and availability of abundant raw materials throughout the year without any supply disruption. Considering these facts, BS could serve as a prominent viable option as raw material in industries. Microbial strain selection and proper choice of appropriate technologies for pre-treatment, fermentation, harvesting and recovery are the best ways to achieve a higher yield of VAP, which also introduces a circular economy to WWTPs [20,21]. Nevertheless, high energy input and operating costs for recovery, minimal market prices and social unacceptability are major obstacles for the development and implementation of BS resource recovery technologies [21]. The recovered resources, however, are odourless and more socially acceptable with high economic value and market demand, and thus, current attention has focused on these techniques [21,22].

BS can be described as a heterogenous matrix consisting of bacterial constituents such as proteins, lipids, cellulose coupled with inorganic matter, other organic matter, pathogens and organic and inorganic pollutants. Disposal of BS with the presence of many biohazardous components, pathogens, and chemical contaminants into the soil via land application/landfilling can lead to widespread environmental problems due to their potential toxicity, carcinogenicity, mutagenicity and ability to be bioaccumulated in the food chain [20]. Specific regulations must be fulfilled when disposing BS for land application. In recent years, different countries have implemented limits on the occurrence of heavy metals, linear alkyl alkynoates, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and phthalates in BS and monitor their impacts while applied on land [23,24]. However, no limits have been proposed for most of the trace organic contaminants (TrOCs) present in BS [13].

TrOCs such as pesticides, industrial chemicals, hormones, PhACs like antibiotics, antimicrobial agents, non-steroidal anti-inflammatory drugs and other organic pollutants pose a major threat as they induce direct biological action in the living system [25,26]. Subsequently, a bioaccumulation of antibiotics in plants has been reported due to a direct application of contaminant-containing BS [25]. A risk quotient evaluation of the common pharmaceuticals found in BS concluded that penicillin, tetracycline, macrolide, quinolone, trimethoprim and sulfonamides pose the maximum risk in the environment, such as hindering the soil ecosystem, inducing antibiotic resistance in microorganisms and affecting soil lifeforms [25]. Moreover, TrOCs which were released into the environment through anthropogenic activities and their ‘natural levels’ continue to increase due to urbanization and industrialization [25,27].

BS containing pathogens and hazardous contaminants affect the soil vegetation and ground water level, so proper treatment and disposal of BS are necessary to protect the environment; this disposal could represent approximately 50% of the total operating cost of WWTPs [3,25]. Moreover BS disposal could be responsible for up to 40% of GHG emissions from WWTPs [28,29]. Meanwhile, BS, which are a considerable source of energy and resources, can be used as an alternative for non-renewable resources, which produce the same amount of energy but have an adverse impact on the environment [6].

The solution for the above-mentioned issues is a sustainable circular economy. The major principle involved in a circular economy is recycling and reuse of products for design and optimization [30]. It replaces the "end of life concept" with restoration, by eliminating the use of toxic chemicals and shifting towards the use of renewable energy and resources [31]. Moreover, reutilizing municipal waste to gain energy or resources and then disposing that waste, has reduced adverse effects on the environment as well as boosted economic growth [30]. In addition, it has created more job opportunities by converting residual municipal waste into high valued resources such as biogas, biopolymers, building aggregates etc., [28].

In the context of a circular economy, VAP obtained as energy from waste can be a substitute for existing energy resources and reduce the associated CO_2 emissions [28]. Sustainable management of BS is about developing innovative technologies to harness the benefits by maximizing waste utilization while considering appropriate social, economic and environmental conditions of the site localization [6]. BS-based VAPs, free of contaminants, are important for protecting biodiversity and addressing the public concern over the beneficial uses of BS [20].

In this paper, solid waste from WWTPs is differentiated into two types: Sludge, concentrated pollutants excreted from sewage water during their purification and biosolids, when sludge meets applicable criteria for their beneficial use. For the past twenty years, work on solid waste management has been growing and this paper summarizes the various resources, which can be recovered from BS and sludge. Furthermore, the paper focuses on volume reduction and TrOCs removal from BS and sludge. This paper also covers the recent developments in waste valorization and VAP production for the period from 2008-2020.

2.2 NUTRIENTS CONTENT AND ORGANIC CONTAMINANTS IN BIOSOLIDS

Depending on the treatment process, BS are usually comprised of 45-70% organic matter with 1-7% nitrogen and ≤ 4% calcium, sulphur, phosphorus, magnesium and potassium, which can serve as an extensive source of nutrients for both plants and microorganisms, and can improve soil quality when applied on the land [11,32,33]. Depending on the application rate, the response

of BS nitrogen is similar to or greater than that of synthetic nitrogen [32]. The concentration of BS phosphorus exceeds crop demands, but its bioavailability is low and has less environmental impact when compared to synthetic phosphorus. The concentration of potassium in BS is low, however, since potassium is highly soluble and is partitioned more into the effluent than into the BS. However, BS have a full source of essential plant nutrients like nitrogen, phosphorus, and potassium, which are helpful in crop fertility [32,33]. The carbon and nutrients contained in BS have value both independently and in combination. Fixed carbon can be used as a source of energy or as a soil conditioner [11].

For several years, there has been a growing concern regarding emerging contaminants of interest in BS and effluents from WWTPs like pesticides, disinfectants, pharmaceuticals, personal care products etc. as well as other inorganic salts also present in BS [34,35]. Several major categories have been reported as substances of concern in BS [3,25]. These include industrial chemicals (pesticides, plasticizers, and alkylbenzene sulfonates, etc.), alkyl phenols, flame retardants, hormones, pharmaceuticals, personal care products, certain metals (arsenic, mercury, silver, and selenium, etc.), polycyclic aromatic hydrocarbons, polychlorinated dioxins and furans, and pathogens [25]. However, their environmental fate and significance is not known or well understood. BS may contain pathogens from industrial and residential wastes [36,37].

The hydrophobic antimicrobial agents, triclosan and triclocarbans, have been reported to be present in effluents and BS in concentrations that pose a threat of bioaccumulation in snails and earthworms [38,39]. Out of the 72 pharmaceutical and personal care products that were tested for in BS, triclosan and triclocarban top the list with concentrations of greater than 1000 mg/kg [40]. The environmental persistence of these agents is mainly due to the incomplete removal by biodegradation in wastewater treatment systems. The endocrine disruptive nature and bioaccumulation in snails and algae proves that the existence of these compounds in the environment is a serious issue [38,41]. Contaminated BS applications on land fields have been reported to induce antibiotic resistance in microbes and also affect earthworms and horsetails [42]. Bisphenol-A (BPA) leaches from the polycarbonate and epoxy resins, and is found in the surface water and BS. On assessing the BPA levels in North America, high concentrations in the range of 0.197-36.7 mg/kg were found in Canada [43]. Though they have lesser half-lives in soil

and are efficiently degraded, the anti-androgenic properties and evidences of their toxicity to plants and invertebrates and their endocrine disruptive nature in mammals have been reported [44,45]. Due to their hydrophobicity, polyaromatic hydrocarbons (PAHs) are emerging as one of the priority contaminants found in soil. PAH degradation in BS amended soil is slow due to their low bioavailability and high molecular weight [46,47]. Limits for PAH concentrations in BS have been assigned in the EU; the maximum allowable concentration is 6 mg/kg [40]. With the increasing energy demand and lack of supply, the need for the sustainable development of these resource-rich BS towards environmentally sound solid waste management becomes unavoidable [48]. The contaminants in the BS, however, can damage the soil ecosystem, pollute the surface water, groundwater and land, contaminate food chains etc., making them unsuitable for disposal without prior treatment. As the physio-chemical nature of the BS is purely dependent on the nature of the wastewater, which may vary with seasonal and treatment processes, the digested sludge must be analyzed properly prior to land applications [11]. BS which have gone through various treatment processes to become solid residue, high in nutrient content and organic matter, can then be utilized for various applications.

2.3 BIOSOLIDS MANAGEMENT AND DISPOSAL

Sludge produced by a WWTP is transported to a sludge treatment line for further treatment; but this process depends on the type and size of the WWTP. The final output of the sludge treatment process is stabilized solid residue containing organic matter, known as BS [49]. However, because of infrastructure and financial restrictions, most developing countries and a few developed countries lack proper sludge management processes [5]. Conventional technologies for sludge management are landfill, incineration, aerobic or anaerobic digestion. Depending on the characteristics of the BS, they can be transported directly to landfills, converted into fertilizer or incinerated [50]. However, lower land availability, operating costs and strict regulations have resulted in processes like landfilling being banned and discouraged in some European and North American countries [51]. However, landfill is mostly applied as a management technique in many developing countries and, incineration in most developed countries [5]. Compared to various technologies, biological processes are economically feasible because of lower energy consumption, low-cost investment, and efficient organic removal rates [52]. Biological treatment

for BS stabilization can be achieved by either anaerobic or aerobic digestion. Indigenous microorganisms in the BS are able to withstand stressful environments and utilize the nutrients, thereby converting these wastes into valuable energy or resources through digestion [5]. Anaerobic digestion is a widely used approach; however, some treatment plants prefer aerobic digestion due to its odor-free operation, effective contaminant removal and enhanced nutrient removal [53].

2.4 PRETREATMENT- AN EFFECTIVE TECHNIQUE FOR ENHANCING SLUDGE SOLUBILIZATION

The handling and disposal of BS is considered to be one of the biggest challenges due to the enormous volume and high moisture content level (80%) of BS. They also contain a substantial amount of biomass, such as carbohydrates, proteins, and lipids, along with various pollutants. [54]. Consequently, the volume of BS has to be reduced using various treatment methods in order to meet the proper disposal standards and to reduce the operational cost of municipal WWTPs [55]. BS biodegradability is limited due to its complex nature [56]. The rigid arrangement of microbial cell walls and membranes also forms a barrier in BS digestion by forming a protective layer which hinders the permeation of hydrolytic enzymes [56–59].

PT techniques were employed to overcome the above-mentioned problem, and fragmentation processes were applied to disrupt the cell walls in the sludge floc structure and push the organic material from the inner layer to the outer layer, making it readily available for microbial degradation during the biological process [5,60]. Enhanced biodegradability, increased organic loading rate, reduced sludge volume and odour reduction are the some of the merits of this PT process [5,61,62]. PT can be either mechanical, chemical, enzymatic or combinations thereof. This part of the section discusses the impact of PT before biological processing of sludge. VAP recovery, contaminant removal and volume reduction that occurred during various PTs of sludge are listed in Tableau 2.1.

During mechanical PT, breakdown of floc particles increases the specific surface area, which subsequently provides better contact with substrate and indigenous microorganisms as a result it

is an enhancement of the anaerobic digestion [63]. The mechanical PT encompasses an extensive range of processes such as ultrasonication, microwave, freeze-thaw, thermal, hydrodynamic cavitation.

Tableau 2.1- Summary of key information of sludge pretreatment prior to biological process

Pretreatment (PT)	**Sludge valorization**			**Reference**
	Solids reduction (%)	**COD solubilization (%)**	**Value added products**	
Mechanical PT				
Ultrasonication			0.35 U/ml of protease and 0.24 U/ml of amylase	[58]
Microwave	9.3	16		[59]
Microwave	17.3	22		
Microwave	24.7	30.2		[60]
Microwave	20.9	30.2		[61]
Thermal		22.3		[63]
Hydro dynamic cavitation		1.4		
Sonication	23	23.2		[65]
Microwave	25.7	33.7		[66]
Ultrasonication		11.1-15.1		[70]
Thermal		≈6-7.9		
Sono-Thermalization		≈17.8-27		
Ultrasonication	22-31			[71]
Thermal	25-39			
Thermal - sonication	29-38			

Ultrasonication			protein	[72]
Thermal			protein	
Ultrasonication	47			[73]
Thermal	5-16			
Microwave		8.5		[75]
Chemical PT				
H_2O_2			protease of 0.123 U/ml and 0.085 U/ml of amylase	[60]
Dioctyl sodium sulpho succinate			0. 04 U/ml of protease and 0. 0.04 U/ml of amylase	[62]
Alkaline		41.6		[63]
Ozonation	13. 4	20.8		[64]
Sodium thiosulphate			0.04 U/ml of protease and 0.03 U/ml of amylase	[67]
MgSO4			0.53 U/ml of protease and 0.19 U/ml of amylase	[68]
KOH			0.15 U/ml of protease and 0.10 U/ml of amylase	[69]
Alkaline			protein	[72]
Ozonation	19			[73]
Potassium ferrate -NaOH	8.11			[74]
Alkaline		18		[75]
Combination of mechanical and chemical PT				
Microwave + $H_2O_2 + H_2SO_4$	35	46		[60]
Microwave + H_2O_2	29.5	50.3		[61]
Microwave +	33	56.1		

$H_2O_2 + H_2SO_4$			
Thermal-Alkaline		46.5	[63]
Hydro dynamic cavitation - Alkaline		44.2	
Thermal-Hydro dynamic cavitation-Alkaline		53	
Ultrasonication - Ozonation	17.8	25.4	[64]
Fenton medicated Sonication	30	34.4	[65]
Rhamnolipid-Microwave	32.6	45.7	[66]
Alkali-Rhamnolipid-Microwave	55.1	42.8	
NaOH-Ultrasonication	13.77.		[74]
Potassium ferrate - Ultrasonication	17.51		
Microwave-Alkaline		46	[75]

Ultrasonic PT is applied as it mechanically disrupts the cell structure and low frequencies of 20 - 40 kHz are effective in sludge treatment [82]. However, high energy input is required for thorough cell lysis [82]. Sonic waves produced during ultrasonication cause rarefaction and periodical compression when propagating through the medium. During this process, microbubbles form which collapse when they reach critical size which in turn initiates the powerful hydro-mechanical shear forces and highly reactive radicals (H· and ·OH). These shear forces and reactive radicals are responsible for the breakup of sludge flocs and release of intercellular materials [83]. Three different sludge PTs, were compared in which ultrasonication is very effective in total suspended solids (TSS) reduction (47%) followed by ozonation (19%) and thermal treatment which was least effective (~5%) [79]. The effect of ultrasonication PT of sludge on extraction of extracellular enzymes such as amylase, and protease from sludge [57,64]. Moreover, no enzyme inactivation was observed during ultrasonication PT.

The major pathway for sludge disruption in microwave irradiation technique, can be explained in two ways: (i) the rotation of dipoles under oscillating electromagnetic fields generates a thermal effect, and as a result, intercellular liquor reaches the boiling point which eventually leads to the break up of bacterial cells and (ii) the changing dipole orientation of polar molecules induces a thermal effect which may break hydrogen bonds and denature biological molecules causing microorganisms to die at lower of hydrogen bonds and denaturing effect, microorganisms dies at lower temperature [84]. Employing microwave irradiation breaks the hydrogen bonds as the polar side chains of larger molecules combine with the path of the electric field. This method requires high energy consumption and is costly, which can be addressed by combining other treatment methods [65]. Microwave PT of sludge at a specific energy input of 18000 kJ/kg TS achieved a COD solubilization and solids reduction of 16% and 9.3% respectively. On the other hand, disperser induced microwave PT of sludge at the same energy input increased the COD solubilization and solids reductions to 22% and 17.3%, respectively [65].

Thermal disintegration is a promising PT for sludge solubilization. Thermal hydrolysis PT processes employ elevated temperatures and pressures in the presence of water. At these high temperatures and pressures, water is able to cleavage of chemical bonds in complex molecules like sludge and convert them into simpler components [84,85]. The effect of thermal, sonication

PT and its combinations, and found that the combined effect of sonic mediated thermal PT achieved a higher degree of COD disintegration (17.8-27%) than individual PT [76].

During the hydrodynamic cavitation process, in which high intensity shockwaves are formed, extreme microjets drive the mechanical and chemical effects. Reflection of these effects results in the release of shear forces and decomposition of water molecules to produce •OH, which subsequently breaks the sludge floc and influences sludge disintegration [86]. The low-level hydrodynamic cavitation (2 bar) assisted thermal-alkaline treatment (50°C & pH = 10) was energy efficient and effective in sludge solubilization, which improved the soluble chemical oxygen demand (SCOD) from 118 mg/L to 10673 mg/L [69]. Moreover, the degree of disintegration (DD_{COD}) improved to 53%, which was 46.5% for low level thermal-alkaline and 1.4% for low level hydrodynamic cavitation, respectively [69].

Chemical PT involves addition of chemical dosage for enhanced solubilization, floc disruption and effective recovery of biopolymers from sludge. The main reaction that occurred during acidic PT was the hydrolysis of hemicellulose, which broke the cell matrix and released monomeric sugars and oligomers [84]. During alkali PT, an increase in pH causes a major reaction such as saponification and solvation. Saponification of particulate organics increases COD solubilization, which can be attributed to repulsions between the negatively charged extracellular polymeric substances (EPS). An increase of this electrostatic repulsion increases negatively charged bacterial surfaces, which causes desorption of extracellular polymers and makes them easily accessible to microorganisms [87].

The recovery of protein from sludge by using three PTs such as ultrasonication, thermal and alkali (chemical), and they found that chemical PT at pH 12.0 is very economic and effective in protein solubilization compared to other PTs [78]. Various chemicals such as H_2O_2, $MgSO_4$, sodium thiosulphate and $MgCl_2$ were also added to sludge prior to biological processes for protease and amylase extraction [66,73–75,88] (Tableau 2.1).

The combined effect of mechanical and chemical PT of sludge on resource recovery are listed in Tableau 2-1. According to the literature, H_2O_2 at a dosage of 0.5 mg/g SS at pH 5.0 (H_2SO_4)

along with microwave (10810 kJ/kg TS) PT, improved the SS reduction and COD solubilization to 35% and 46%, respectively [66]. Surfactant mediated microwave extraction in alkaline conditions improved the COD solubilization and solid reductions compare to individual PT [72]. The combination of two chemical agents such as potassium ferrate and NaOH in sludge and observed that volatile suspended solid (VSS) reduction was around 8% [80]. The impacts of ultrasonication of the chemically treated sludge were also examined; mechanical-chemical PT incurred high VSS removal efficiencies (13.77% for NaOH + ultrasonication; 17.51% for potassium ferrate + ultrasonication). However, adding ultrasonication to the combination increased costs by 45-66% [80] (Tableau 2.1).

Whether it was mechanical, chemical, enzymatic or combined PT, sludge disintegration was equally effective, but contaminant removal and VAP production were less effective. Disruption of sludge flocs, effectively released the EPS, however, the macromolecules released along with contaminants were available in the liquid phase, which implies that these components were not removed but readily available for degradation in another manner [25,26]. So, contaminant removal or resource recovery will be improved when this PT is further accompanied by biological processes.

2.5 PRETREATMENT ASSISTED BIOTECHNOLOGICAL PROCESS - AMALGAMATED STRATEGY FOR RESOURCE RECOVERY AND SLUDGE STABILIZATION

In aerobic digestion, nutrient-rich BS are converted into energy or bioproducts and suitable organic residue is obtained [5]. However, in the biological process, hydrolysis is the rate-limiting step because extracellular enzymes produced by indigenous microorganisms are immobilized inside the floc, which makes them unavailable for the hydrolysis of readily available and large organic matter, which in turn affect the stabilization of BS [3]. Hence, to aid in the solubilization of organic matter for biodegradation and disintegrating the sludge, several PT methods, such as mechanical, chemical, thermal and biological [89] or a combination thereof are required prior to aerobic treatment. Use of these PT methods accelerates the rate of hydrolysis, and improves the dewaterability of sludge while reducing pathogens [90]. PT made substrate more accessible to

the indigenous microbial consortium, which accelerated the digestion process as demonstrated by the improved degradation and reduction in sludge volume. PT applied to sludge prior to aerobic digestion converts harder to degrade complex organic matter into more easily degradable compounds and releases them into the soluble phase. These organic molecules present in the soluble phase are eventually available for biodegradation. Moreover, PT followed by subsequent biological processes achieves improved sludge degradation, enhanced biogas production, VAP extraction, reduced retention time in digesters and lessens the cost of sludge disposal [51]. The following section discusses the importance of aerobic digestion, which is assisted by PT. Moreover, it will show the impact of PT over aerobic digestion on production of VAPs (Tableau 2.2).

2.5.1. Aerobic digestion

Three different PTs, ultrasonication, ozonation, and thermal, were used prior to aerobic digestion. TS reduction and aerobic biodegradability were monitored to assess the performance of PT [91]. TSS removal improved by 15% and 5% for ultrasonication and thermal assisted aerobic digestion. On the other hand, no TSS removal was obtained with ozonation PT assisted aerobic digestion. This might be explained by the release of radical scavengers, which are inefficient at materializing the sludge because of high ozone dosage (>0.1 g O3/g TS). Aerobic biodegradability can be expressed by three factors i) global CO_2 production ii) maximum specific CO_2 production rate and iii) initial CO_2 production rate. The global CO_2 production, initial CO_2 production rate and maximum specific CO_2 production rate improved to 19%, 11% and 3.4% respectively for thermal assisted aerobic processes, nil, 2% and 30.3% respectively, for ultrasonication assisted aerobic processes and 5%, 10% and 20% respectively, for ozonation assisted aerobic processes. Based on the global CO_2 production, sludge degradability improved for thermal and ozonation PT. But, no real improvement in biodegradability was observed for sonication assisted aerobic digestion [91]. Combining microwave and alkali PTs improves the rate of solubilization while reducing the energy consumption and reaction time when compared to microwave or alkali PT alone. A 63% reduction of VSS was observed for aerobic digestion of sludge by integrated microwave and alkali PT [81].

Tableau 2.2- Summary of key information of sludge pretreatment assisted aerobic digestion

Type of PT	Sludge valorization			Remarks	Reference
	Solids reduction (%)	Contaminants removal (%)	Value added products		
Ultrasonication Thermal Ozonation	76% 62-68% 71%			Cost reduction can be effective when ultrasonication and ozonation prior to digestion.	[73]
Ultrasonication Ozonation	36% 34.1%			PT with ultrasonication increases the sludge resistance to dewatering	[87]
Free ammonia	36± 4%			BS disposal and transportation cost is higher than $40/wet tonne, free ammonia PT will be economically feasible.	[88]
Thermal	5%		Biogas production	Aerobic digestion	[86]

			improved by 19%	followed by thermal PT	
Ultrasonication	15%			improved BS	
Ozonation	-		Biogas production improved by 5%	reduction and biodegradability compared to other two treatments.	
		Azithyromycin -50%, and citalopram-10%		PhACs degradation efficiency increased by changing C/N ratio	[89]
		Pharmaceuticals (42-100%)	No laccase activity	Compared with autochthons microbiome, BS bioaugmented with *Trametes versicolor* has higher removal of PhACs	[90]
		21 pharmaceuticals (reduced to 70 µg/kg dm from 142 µg/kg dm)		Lower decrease is obtained for Aerobic + dehydrated sludge compared to Anaerobic + dehydrated BS	[91]

		89.6–95.4% of norflaxin and 87.2–95.4% of ofloxacin	Biodegradation is the major pathway for PhACs removal which was followed by irreversible radiation and volatilization	[92]
		95% degradation of diclofenac	1:3 ratio of BS and sawdust has higher removal compared to 1:1 ratio	[93]
Microwave-alkali	63%		Combined PT improves solubilization rate, reduces energy consumption and reaction time compared to the individual PT	[75]
		Metformin-90%	No removal occurred for carbamazepine during composting	[94]

Free ammonia PT with subsequent aerobic digestion for 15 days, which resulted in a 36% volatile solid reduction with improved biodegradability [93]. Degradation in the first 6 days of the 15 days of the aerobic digestion was faster since it occurred in the more easily biodegradable molecules first. After 6 days, the degradation was slow, and it becomes stabilized. However, PT becomes practically feasible when BS disposal and transportation costs are higher than US$40/ton of dried BS [93].

Ultrasonication, ozonation and thermal PT with subsequent aerobic and anaerobic digestion were tested separately, and ultrasonication or ozonation PT assisted biological processes were found to be energy efficient and cost effective. PT of anaerobic waste resulted in higher TSS reduction in contrast to aerobic waste, implying that anaerobic treatment is effective when compared to aerobic treatment during BS reduction [79].

A degradation study on diclofenac was carried out on digested dewatered biosolids. During this study anaerobic digested sludge was mixed with sawdust in two different ratios of 1:2 and 1:3, followed by aerobic composting. Over 95% removal of diclofenac was observed in both compost mixtures. However, the PhACs degradation was comparatively higher in the 1:3 compost mixture [98]. Ten different commonly occurring PhACs and their degradation by composting of sludge with enrichment (rice straw) were analyzed. Reduction due to composting (C/N:20) occurred for azithromycin, irbesartan, fluoxetine, and citalopram but telmisartan and venlafaxine showed no signs of degradation. Composting with five different C/N ratio blends (C/N:17; C/N:20; C/N:24; C/N:29; C/N:37) for PhACs removal were also examined. Five out of 10 PhACs, azithromycin, ibuprofen, irbesartan, olanzapine, and benzylpenicillin, were reduced in all the composting studies. However, telmisartan was only reduced in C/N:37 and fluoxetine, venlafaxine, and citalopram were only reduced in C/N:20. The trend shows that the degradation effect of PhACs depends on different composting blends with different half-life periods. Microorganisms use azithromycin and ibuprofen as carbon sources and irbesartan as a sole nitrogen source during degradation and composting [94] (Tableau 2.2). PhACs concentrations in dehydrated sludge were reduced more in anaerobic digestion than in aerobic digestion [96]. Biodegradation followed by irreversible radiation and volatilization alleviated pharmaceutical removal for aerobic treatment [97].

2.6 BIOAUGMENTATION- A REINVIGORATING APPROACH TO PRETREATMENT ENHANCED DIGESTION FOR SLUDGE VALORIZATION

Environmentally friendly approaches such as adding enzyme secreting bacteria (bioaugmentation) and/or pretreating with enzymes (biostimulation) have the potential to reduce the volume of sludge and simultaneously remove pollutants contained in it [20,26,100]. The global market for waste management was valued at around US$2080 billion in 2019, with an estimated compound annual growth rate (CAGR) of 5.5% between 2020 and 2027 and is expected to reach US$2339.8 billion by 2027 [101], with bioaugmentation representing about a ninth of it. Bioaugmentation is a technique to increase the rate of bioremediation by taking advantage of the synergistic effect among organisms. A successful bioremediation process usually involves the use of approaches tailored for the specific environmental conditions at the site. The need for bioaugmentation arises when the indigenous organisms are slow/unable to biodegrade the contaminants in the sludge. The introduction of new strains improves the performance of the existing strains while simultaneously boosting contaminant removal. In addition, if appropriate formulations are applied, the sludge microbial mixture can be used as microbial inoculants or biopesticides for agricultural land [20,21]. However, the major setback associated with this biological approach is time consumption and proper selection of microorganisms/enzymes. Inaccessibility of substrate, where the polymeric substances immobilized within sludge floc make things difficult for bioaugmented strain/indigenous microbes, could be the cause of this time consumption. The rate-limiting step of hydrolysis can be avoided by introducing PT prior to bioaugmentation. Mechanical, chemical or their combinational PTs are well known for their merits such as odourless sludge, extraction of VAPs and the removal of pollutants. However, the major demerits are environmental pollution due to chemical agents, economic constraints and operational feasibility [25,26,100,102]. So, the next part of this section focuses on the impact of introducing PT to bioaugmentation and its potentially prolonged effect on the system. In addition, it provides an overview of which PT are suitable for aerobic digestion and how it is effective over VAP production and its associated costs.

While the bioaugmentation technique is theoretically feasible, in practice, there are numerous obstacles such as the availability of substrate, competition between microbes, preference of organic substrates over pollutants, and predation [103]. The abiotic stresses that suppress microbial growth include water content, temperature, nutrient depletion, pH, and potentially toxic pollutant levels in the biosolids [104]. These constraints in addition to insufficient published literature have a negative effect on the trials and laboratory experiments. Some of the key factors of this negative effect are microbial selection, fermentation environment, contaminant type, abiotic parameters, and issues related to introduction [105]. The microbial selection is usually done by adding a microbial strain, microbial consortium or by introducing pre-adapted genetically engineered microorganisms.

Despite the challenges mentioned above, bioaugmentation has shown various applications in soil remediation [106], oil spills [107], WWTPs [108] and ground water de-chlorination [109,110]. Most of the reviews about bioaugmentation in soil remediation during the late 20^{th} century were of a cautionary tale [111]. This was mainly due to the insufficient literature and debilitated lab-scale experiments. Moreover, resource recovery was not considered, and the contaminant removal associated was low. However, this has changed over the years and with increasing information about interaction of organisms and the genetic predisposition has led to a tremendous increase in scope [112]. The use of bioaugmentation over other methods is attributed to its productivity and its cost-effectiveness [113]. Specifically, PAHs can be metabolized by a consortium of bacteria, which prevents the environment by avoid leaching of these contaminants into groundwater. In areas where groundwater is contaminated by chlorinated ethenes [114], bioaugmentation ensures that the *in situ* microorganisms completely degrade the chemicals to chlorine and ethylene [115].

Typically, it is used only in the bioremediation of chlorinated ethenes although research is being done in compounds like BTEX, chloroethanes [110] chloromethanes and MTBE [116] Valorization of biosolids in bioaugmentation has three main criteria. First, the massive volume of biosolids is a key criterion when biodegradation of waste is concerned. Thus, volume reduction is essential for proper disposal of the waste. Second, toxic by-products in waste-generating plants

could lead to bioaccumulation. The waste should therefore be devoid of toxic contaminants before disposal [117]. Finally, microbial activity may lead to the formation of VAP, like surface active compounds, bioactive compounds, phytochemicals and dietary fibres [118]. This "resource recovery" helps in the circular economy, and hence, forms an essential part of waste valorization. In addition, the cost and energy consumption of the methods play a major role during their use in large volume bioreactors [119].

Various microbes had been used in bioaugmentation like *Bacillus spp.* [64,68,120], *T. versicolor* [95,121,122], *Bjerkandera adusta* [123], *Bacillus licheniformis* [124], *Bacillus thuringenesis* [125–128] and *Exiguobacterium* sp. [75] (Tableau 2.3). A major factor to be considered before bioaugmentation is the type of fermentation. The VAP to be obtained depends greatly on the organism and its metabolism. Augmenting aerobic organisms during anaerobic fermentation will result in failure and vice-versa. Additionally, aerobic, and anaerobic fermentation have different effects on waste, especially activated sludge. This is evident in the work done by [64,88,89,120] where they bioaugmented *Bacillus jerish* 03 and *B. jerish* 04 in both aerobic and anaerobic conditions. This served as an appropriate barometer to compare the two types of fermentation.

2.6.1 Aerobic Digestion

The sludge was treated under aerobic conditions by bioaugmenting *B.jerish 03* and *B. jerish 04* which were previously assisted by either ultrasonication or ethylene diamine tetra acetic acid (EDTA) for EPS release [64,120]. The reduction in SS, and COD solubilization were evaluated to consider the effect of the volume reduction and biodegradability of the sludge. Furthermore, VAPs, like protease and amylase, were quantified. To evaluate the importance of ultrasonication PT, ultrasonication assisted bioaugmentation, and bioaugmentation without ultrasonication were performed [64]. The complex nature of the flocs and immobilization of the enzymes inside the flocs restricted the disintegration potential in the bioaugmentation without ultrasonication, which had a 15% SS reduction after 56h. On the other hand, SS reduction in PT assisted bioaugmentation was about 21%; floc disruption during ultrasonication had helped the bacterial consortium along with bioaugmented bacteria to achieve improved SS reduction. The energy consumption and associated costs were calculated to determine the proficiency of the method. PT assisted bioaugmentation consumed 160.8 kWh of energy and earned a net profit of US$27/ton

of dried sludge, compared to 160.2 kWh and a negative net gain (-US$6/ton of dried sludge) bioaugmentation without PT [64]. The impact of adding EDTA prior to bioaugmentation of *B. jerish 03* and *B. jerish 04* for the degradation process was studied by [120], where SS reduction for EDTA-assisted bioaugmentation was 24% versus 15.7% for bioaugmentation without EDTA addition. Enhanced 8.3% removal for EDTA-assisted bioaugmentation was supported by combined enzyme activity (enzymes secreted by facultative anaerobes along with extraction of enzymes from the sludge matrix by EDTA). While, at the end of the experiment, the SS reduction of EDTA-assisted bioaugmentation was 48.5%, which was 34.3% higher than for the control (raw sludge) [120].

Tableau 2.3- Summary of key information on pretreatement assisted bioaugmentation aided aerobic digestion

Type of PT	**Bioaugmentation**	**Sludge valorization**			**Remarks**	**Reference**
		Solids reduction (%)	Contaminants removal (%)	Value added products		
Ultrasonication	*B. jerish* 03 & *B. jerish* 04	20.7		Maximum enzymatic activity of 0.15 U/ml of protease and 0.12 U/ml of amylase	Net profit of 27 USD/ton of sludge was obtained with ultrasonic aided bacterial disintegration	[58]
	B. jerish 03 & *B. jerish* 04	14.3		Maximum enzymatic activity of 0.07 U/ml of protease and 0.06 U/ml of	sludge disintegrated only with help of bacteria earned a negative net	

				amylase	cost (-6 USD/ton of sludge).	
Citric acid	*B. licheniformis*	18			Citric acid mediated bacterial disintegration is cost effective (₹0.24/day) when compared with only bacterial disintegration.	[119]
	B. licheniformis	10				
	B.thuringenesis			Biopesticides (max spore count-8.15±0.04 (107) CFU g/DM.	However, with different treatment strategies, production yield of value-added products were low.	[47]
	Starmerella bombicola			Biosurfactant maximum yield (Sophrolipids = 0.02 g /g-DM		
	Ensifer sp		Acetaminophen removal		Compared to autochthonous microbiota,	[124]

			Acetaminophen was degraded in less than 1 hr for *Ensifer* bioaugmented samples which is 22 hrs for control	
	B. thuringiensis	Viable Cell count- 1.44×10^8 CFU/ ml Sporulation rate-96%	Fenton is more effective than ultrasonication	[121]
Fenton oxidation	*B. thuringiensis*	Viable Cell count- 1.63×10^9 CFU/ ml Sporulation rate-90%		
Ultrasonication	*B. thuringiensis*	Viable Cell count- 4.08×10^8 CFU/ ml Sporulation rate-84%		
Insulated	*B. thuringiensis*	Sporulation rate-84% Viable cell	All three experiments posses high	[125]

			count- 10^9 CFU/ g DM	sporulation rate and viable cell count	
Non-Insulated	*B. thuringiensis*		Sporulation rate-97% Viable cell count- 10^9 CFU/ g DM		
Non-Insulated and stirred	*B. thuringiensis*		Sporulation rate-89% Viable cell count- 10^9 CFU/ g DM		
Sterilization	*T. versicolor*	Sulfonamide-100%		Complete elimination of sulfonamides at real environmental conditions.	[126]
	T. versicolor	Pharmaceuticals (56-100%)	Maximum laccase activity of 4.58 U/g DW	Compared with autochthons microbiome, bioaugmented with *T. versicolor* has higher removal of PhACs	[127]

	T. versicolor	Pharmaceuticals (86%), Brominated -flame-retardants (81%) and UV filters (80%)		Reinoculation has improved the removal rate compares to first inoculation.	[117]
Sterilization	*T. versicolor*	Pharmaceuticals (43-100%), Polychlorinated biphenyls (3.8-99.9%)	Maximum laccase activity of 3-4 U/g DW	Reduction in sludge toxicity after treatment	[128]
Sterilization	*T. versicolor*	Pharmaceuticals (66%),		Fungal colonization was observed in first half and its reduced at fag end of experiment.	[116]

Contaminant removal in addition to bioaugmentation has been studied extensively with *T. versicolor* [134]. Complete removal of sulfonamide under environmental conditions was observed with *T. versicolor*. 3.8-99% of PCBs and 26-100% of PhACs were removed under environmental conditions [95,133]. They also showed that the removal of PhACs along with the production of laccases were higher in fungal bioaugmented sludge. Out of 45 PhACs

investigated in the biopiles, only 19 were detected [121]. They studied the effect of reinoculation of *T. versicolor* at the middle of the period. The percentages for the total removal of PhACs after the end of the 42-day experiment of non reinoculated and reinoculated samples were 49.2% and 66.5%, respectively During this study, they also analyzed microbial diversity in the biopiles system, and found that *T. versicolor* fungal colonization was predominant during the first half of the experiment (i.e., up to 23 days), but was no longer predominant by the end of the study. PhACs removal had improved in the re-inoculated biopiles. Moreover, a change in the fungal population hadn't affected the bacterial populations, which accelerated the contaminant removal [121]. VAP obtained from the sludge valorisation using aerobic bioaugmentation were listed in Tableau 2.3. The promising VAP are biodiesel from *Trichosporon oleaginosus* [135,136], enzymes from *A. niger* [12], *T. versicolor* [121,122,133] and *B. subtilis* [53], biopesticide from *B. thuringiensis*, [125,137] and biosurfactant from *S. bombicola* [125]. These products have future applications in extraction and production, and thus, provide a way for waste disposal and cost-effective production of VAPs.

2.7 BIOSOLIDS REUSE AS RESOURCES - A BIOREFINERY PERSPECTIVE FOR THE PRODUCTION OF ADDED-VALUE PRODUCTS

2.7.1 Biodiesel

The current trend of our ever-increasing global population and its use of petroleum-based products predicts the depletion of these energy resources at a rapid rate. Moreover, environmental threats related to the use of these chemicals has led to the creation of an alternate pathway for the production of biobased products to alleviate GHGs and fulfill the global demand [59,138,139].

Municipal sewage sludge, a potential feedstock, which consists of high lipid content and is available at zero cost, can be a viable alternative to producing biodiesel [140,140]. Sewage sludge, which has been reported to contain 5–20% lipid w/w dry sludge [59,141] and is rich in nutrients, can be used as the medium for microorganisms for lipid production, which constitutes an efficient way to reduce the cost of biodiesel production [59]. Studies on biodiesel production

in addition to bioaugmentation have been done extensively on oleaginous yeast species [59,135,142–144].

Microorganisms, which can accumulate more than 20% (g/g) lipids, are known as oleaginous microorganisms. For lipid accumulation in microorganisms, a high C/N ratio is required. In environmental conditions like sludge, lipid accumulation for microorganisms have been improved by introducing PT methods [145]. Municipal sludge with crude glycerol and NH_4Cl was used for the production of lipid content by *T.oleaginosus.* After 60 h, the lipid content and lipid concentration were 43.77% (w/w), and 22.32 g/L, respectively. Moreover, the final estimated production cost of biodiesel was US$630/ton of dried sludge, which was more competitive than the commercial biodiesel production cost (US$900/ton of dried sludge) [135]. The thermo-sonic enzymatic PT of sludge with the subsequent addition of *Naganishia liquefaciens* for biodiesel production [142]. The addition of yeast to thermal-sonic assisted enzymatic PT (TSEP) improved the lipid yield to 65.4%, which was ten times higher than the undigested medium, and can be attributed to an adequate amount of nutrient release during TSEP [142]. Various PT assisted bioaugmentation, such as sonication [144], thermo-chemo-sonic [143], chemical treatment [136], to produce lipids for the biodiesel production were studied (Tableau 2-4). *Lipomyces starkeyi,* an oleaginous yeast was reported to store a large amount of lipids in sludge media compared to other yeast considered for studies. Sonication prior to bioaugmentation was found to be a potential technology for lipid extraction since sonic irradiation, which causes bubbles to collapse, results in cell lysis as well as the release of lipids [145]. For a higher yield of biodiesel production and lipid extraction from sewage sludge, primary sludge, and scum sludge, which contain more lipids, are potential feedstock compared to secondary sludge. However, for better biodiesel yield, a profiling database for sludge composition from the WWTPs is required [146].

Tableau 2.4- Summary of key information on VAP production by biological process

Sludge/pretreatment	Organism	Yield	Operating parameters	Reference
Biopesticides				
Digestate	*B. thuringiensis*	Viable spore count (VSC) - $8.15x10^7$ CFU g/DM	180 rpm, 30°C, 20h	[47]
Sludge	*B. thuringiensis*	Sporulation rate (SR) - 88-94%	pH 6.0, 34 – 36 °C	[143]
Sewage sludge	*B. thuringiensis*	SR 38-47%	pH 7.0, 34°C, 84h	[144]
Sludge	*B. thuringiensis*	SR 95%	pH 7.0, 30°C	[145]
WAS	*B. thuringiensis*		pH 7.0, 25 - 30°C	[146]
Sewage sludge	*B. thuringiensis* *B. sphaericus*	VSC - 2.1×10^9 6.8×10^8 CFU/mL	pH 7.0, 30°C, 500 rpm	[147]
Secondary sludge	*B. thuringiensis*	SR 14-91%	pH 6.0, 28◦C, 250 rpm, TS 10–50 g/L	[148]
Organic sludge	*B. thuringiensis*	VSC - $1.0x10^6$ CFU/g	pH 8.36, 6 days ,65°C	[149]
Dewatered sludge (95 °C Pasteurization for 2 h)	*B. thuringiensis*	25 g/L VSC 5.8×10^8 CFU/ml	pH 7.5, 30 days, 30 °C C/N Ratio 9.9 Sludge solids 25 g/L	[123]
Secondary WAS	*B. thuringiensis*	8.6×10^8 CFU/cm^3 95% SR	pH 8.2, 36 h, 30°C	[150]
Bioflocculant				
WAS (alkaline	*Rhodococcus*	4.2 g/L	10, 60 h, 25°C	[151]

thermal pretreatment)	*erythropolis*			
Excess biological sludge		4 g/L	500 rpm, 35 min	[152]
WAS (sterilized, alkaline and thermal)	*Serratia sp*	1.5–3.4 g/L	25◦C, 72 h, 250 rpm, Inoculum size 3% (v/v)	[153]
Bioplastics				
Sewage sludge and organic fraction of municipal solid waste		6.5% wt	22-25°c, pH 8-9	[154]
Secondary sludge from clarifier tank		6.04%	70 °c,72 hrs	[135]
Waste activated sludge	*Zobellella denitrificans*	0.38-0.56 g/L PHB	pH-7.3	[155]
Enzymes				
Waste activated sludge	(*B. jerish* 03 along with, *B. jerish* 04)	protease and amylase	pH 6.5, temperature 40 C with a shaking speed of 150 rpm for 42 hr	[115]
Activated sludge (Ultrasound, EDTA)		protease & lipase	500 rpm at different extraction times, temperature 5 ± 1 C.	[156]
Wastewater sludge	*Trichoderma*	Cellulase	pH 4-6, 30-	[157]

	harzianum		35°C,100-250 rpm	
Activated sludge (Ultrasound, EDTA)		Protease,α Amylase,α Glucosidase, Alkaline phosphatase, Acid phosphatase	7.0, 4◦C ,3 h , 20–40 kHz, 2–20 min, 138–690 W/g VSS, EDTA 2%	[78]
Waste activated sludge (Ultrasound, Triton X 100)		Protease, Lipase	24 kHz, 3.9 W/cm2, 20 min, 5±1◦C, and Triton X100- 0.1 to 2% (v/v)	[158]
Municipal biosolids		Glucose oxidase	72 h; Inoculum size-20% (w/v)	[10]
Biodiesel				
Secondary sludge (Ultrasonication)	*T. oleaginosus*	95%	20–25 °C , (5–60 min	[140]
Secondary sludge (Chemical-treated)	*T. oleaginosus*	Lipid content-39%	6.5, 121 °C for 15 min	[131]
Sewage sludge	*T. oleaginosus*	Lipid content-39%	30 °C, DO>30% (v/v)	[130]

WAS (thermo-sonic assisted enzymatic)	*Naganishia liquefaciens*	Lipid content-65.4%	5-50% (v/ v), (30–50 °C) , (200 rpm) , 40 h	[138]

2.7.2 Bioplastics

The demand for plastics is ever increasing due to their application in various fields including packaging, construction, medicine, agriculture, electrical and automotive. But the detrimental effect of plastics on the environment is massive [162,163]. This global issue warrants an eco-friendly alternative for conventional plastic materials. Bioplastics produced from renewable biomass can sizably shrink the environmental ramifications of plastic usage. Polyhydroxyalkanoates (PHAs) and their derivatives are one of the major types of biodegradable and biocompatible bioplastics [164]. In the presence of sugar and lipids in the natural environment, some bacteria can produce PHAs, which are analogous to conventional plastics and can be used as an alternative to petro-plastics. Moreover, products from microbial fermentation are biodegradable, which helps to protect the environment and human health. Municipal sludge, which is mainly comprised of carbohydrates and lipids, can be seen as a viable option for the production of bioplastics [165]. Among the various biorefinery-based products, the rapidly growing commodity is bioplastics and the CAGR of bioplastics will increase by about 16.6% by 2025 [138] (Tableau 2.4). In anaerobic digestion, during acidogenic fermentation when the methanogenic step is inhibited, waste activated sludge can be converted into volatile fatty acids (VFAs). VFAs with lower carbon mostly follow three different pathways, condensation, de nova and polymerization to convert the two acetyl CoA to PHAs in the presence of PHA synthase enzyme [166]. PHAs content in WAS has been reported to range from 0.3 to 22.7 mg [167].

Bioaugmentation of *Zobellella denitrificans* in sludge under saline conditions for the production of PHB [159]. C/N ratio, electron acceptors and saline factors affected the growth of *Z. denitrificans* and subsequent production of PHB. Increasing the saline conditions from no NaCl to 3% NaCl, the PHB production increased to 0.56 mg/L from 0.38 mg/L [159]. Mixing organic municipal solid waste and sewage sludge in a three-step mixed microbial culture (MMC) was investigated. In a fed batch test, MMC storing PHAs was able to accumulate 46% wt of PHAs.

However, while considering the mass flows in each process step and full-scale application, the overall yield of PHA was about 6.5% wt [158]. Various parameters of secondary sludge were optimized using RSM, and a yield of about 6% PHA was achieved, where the mcl PHA (58%) was found to be dominant along with various PHA [139] (Tableau 2-4).

2.7.3 Bioflocculants

Like bioplastics, bioflocculants are also naturally secreted by bacteria during their growth. Chemical flocculants or polymers, which are mostly used in wastewater treatment plants for coagulation-flocculation processes, are corrosive and toxic, and can cause adverse effects to the environment [168]. Microbial flocculants, which are biodegradable can be used as a replacement for conventional polymers for a safe environment [157,169]. However, the expensive substrate cost, which impacts the overall production cost, is the major limitation for their practical application. Biosolids, a microbial rich source, which contains macromolecular compounds with flocculating activity, makes waste a viable alternative option for high cost substrates for industrial applications [155] (Tableau 2.4).

Three different PT biosolids (sterilization, alkali-thermal and acid-thermal) were used as raw material for *Rhodococcus R3* and *Serratia sp* inoculation by [151] and [153] respectively. Bioflocculant production in acid-thermal PT was low when compared with sterilization PT as well as alkali-thermal PT. The release of nutrients and their availability in medium, cell lysis and growth inhibition during the PT can be attributed to the production of bioflocculant. Bioflocculant production results in the release of nutrients and their bioavailability in the medium, and cell lysis and growth inhibition during the PT [155]. Moreover, bioflocculant stability of alkaline thermal bioflocculant was investigated at varying temperatures and by adding enzymes. The flocculating rate decreased with an increase in temperature, and it only maintained up to 90% of its rate at relatively low temperatures (60°C for 30 min). When the temperature was increased further to 80°C, the rate decreased to 50%. Addition of amylase, cellulase, glucoamylase, and glycosidase did not reduce the flocculation activity, which indicated that bioflocculant is neither a polysaccharide nor a glycoprotein. However, hydrolysis by pepsin and trypsin reduced the flocculation rate confirming that the bioingredient of the obtained

flocculant is protein [155]. Addition of HCl to sludge, and tested factors such as pH, dosage, and temperature to optimize the bioflocculant production [156]. The flocculating rate was high in the pH range of 5-11, at a dosage concentration above 1% and temperature less than 40°C. They also used NaOH and ethanol for purification and compared to ethanol, separation efficiency was effective with sodium hydroxide (Tableau 2.4).

2.7.4 Enzymes

Enzymes, which increase the rate of a chemical reaction, have a commercial significance and are used for industrial applications. According to business communications company (BCC) research, the CAGR value of enzymes was expected to be 4.9% for the period of 2018 to 2023 [170]. Enzymes account for 30-40% of the production cost for the preparation of culture medium in industries [171,172]. The organic matter of sludge is mainly comprised of carbohydrates, proteins, lipids, and enzymes such as amylase, protease, lipase, glycosidase and aminopeptidases, which play a part in biodegradation [3,173]. So, its recovery is of high importance because of its use in various industrial applications such as pharmaceuticals, detergents, and food industries. Moreover, the recovered resource is from no/low cost waste, which reduces the industrial processing cost [172]. The vast microbial consortia present in sludge produce enzymes and release them into the medium to degrade the organic matter of sludge. Glucose oxidase (GOD) is one of the most important enzymes that can be produced by using this nutritious biomass from sludge. A cost-effective process to produce GOD using BS as a substrate by employing *A. niger* [12]. A maximum GOD activity of 6012 U/L was achieved in 48 h when 72-h old inoculum of 20% (w/v) inoculum size was used in 25% (dw/v) BS media. This approach can also simultaneously improve recalcitrant organic compound removal in treatment plants [12].

The recovery of amylase and protease enzyme activities while adding EDTA prior to the addition of bacteria was studied. Enzyme activity in EPS-removed sludge improved to 0.18 U/ml from 0.08 U/ml for non-EPS removed sludge. The increase in enzyme activity may be due to the effective release of carbon and nitrogen sources as a result of the EDTA addition and subsequent utilization of nutrients by bacteria in the reactor [120]. Addition of Triton X100 along with

ultrasonication of the sludge for the extraction of lipase and protease, and further used ammonium precipitation, dialysis and lyophilization as a purification procedure for the extracted lipase enzyme [161]. Adding Triton X100 (0% to 2% v/v) to ultrasonic disintegration had a remarkable impact on the extraction of protease (0 to 52 protease units/g VSS) but, a negligible impact on lipase extraction (near constant and equal to 21 lipase units/g VSS). The extracted lipase underwent various purification studies, and of these studies, maximum recovery was obtained with ultrasonication + 0% Triton X 100 + precipitation + lyophilization (73.9%) followed by ultrasonication + 0% Triton X 100 + dialysis + lyophilization, ultrasonication + 0% Triton X 100 + lyophilization [161] (Tableau 2.4).

2.7.5 Biofertilizers

The residual BS obtained after sludge treatment are a rich source of organic and inorganic plant nutrients and may be a realistic substitute for fertilizers [174]. N-fertilizers originating from waste sludge and livestock manure could be a viable option for partially fulfilling manufactured fertilizer requirements, thus reducing its energy and resource footprint [175]. Biosolids has microbial biomass that store beneficial amounts of nitrogen and phosphorus suitable for crop growth [176]. Sludge composts have nitrogen restoring capacities and can increase water retentivity in soil while circumventing non-point source pollution incurred by the use of commercial fertilizers [177]. Various advancements in processes that convert different organic waste streams, including municipal solid waste, into biofertilizers were reviewed and field-tested by [178].

Aerobically digested sewage sludge yielded acceptable nitrogen fertilizer replacement value (NFRV) results compared with mineral reference treatments whereas the anaerobically digested sewage sludge required some additional N [176]. Biofertilizer production approach by co-digestion of activated sludge and fungal mash hydrolysed-food waste and described its economic feasibility [179]. Ash, a by-product obtained by incinerating dewatered biosolids, was identified as a potential substrate to grow *Rhizobium*. It can be used as inoculum to formulate biofertilizers with the capacity to induce high germination and nodulation in crops [180]. Application of phosphate sludge (i.e., sludge generated during the treatment of phosphate rock; many of the minerals removed constitute a sludge with phosphate in the insoluble form) supplied with

phosphate solubilizing bacteria (PSB) to promote *Zea mays* plant growth [181]. The synergistic interaction of the microbial consortia of PSB could improve siderophores, HCN, H_2S, and most importantly Indol-3-acetic acid production. It could also increase the solubility of P for easier plant uptake [181]. The produced siderophores can also increase soil fertility and act as a biocontrol for fungal pathogens [182]. On the other hand, the presence of TrOCs in biosolid-amended soil, could lead to the uptake of these contaminants into plants and subsequent possible risk of human exposure [183]. However, the vegetable harvested at the control site were high in TrOCs concentration, the risk of human exposure to those TrOCs produced from biosolid-amended soil was low [184,185]. Plants uptake TrOCs from biosolid-amended soil by passive absorption of soil water through the epidermal layer root and into the root tissue cortex. These contaminants are then transported to other parts of the plants via simple diffusion and transpiration through xylem [183]. The bioaccumulation of TrOCs in vegetable [185], and earthworms [186] via biosolids amended soil were observed.

2.7.6 Biopesticides

Biopesticides, a non-toxic residue to invertebrates, has a minimal impact on the environment compared to chemical pesticides. Biopesticides have the potential for effective pest control through bioactive microbial activity [187] to increase the yield and quality of crops while circumventing the non-target toxicity and detrimental environmental impact incurred by the use of synthetic pesticides. Biopesticides have an added advantage of having a complex mode of action thereby delaying the resistance adaptation of the pests [188]. According to the US market, the CAGR value of biopesticides will be 17% between the period of 2016 and 2022 [189]. *B. thuringiensis,* bacteriophages and *T. viride* are some of the widely used species in biocontrol applications. Almost 90% of all biopesticide production experiments have been performed using the ubiquitous aerobic *B. thuringiensis* (Bt) [190], which is known to be able to produce delta endotoxins (δ-endotoxin) during sporulation. The latter is widely used in agronomy, forestry and the public health sectors [172,191]. However, the cost associated with the use of raw material for Bt inoculation is a major obstacle for its commercial application [172].

Biosolids can be used as a culture medium for Bt to produce value-added pesticides in a mineral rich culture medium. Bt inoculation in two bench-scale (10 L and 22 L) and one pilot-scale

reactors (100 L) were studied by [127] in insulated, non-insulated, stirred, and non-stirred conditions. In a non temperature-controlled reactor (10 L), the viable cell count and spore counts increased from the beginning to reach a maximum at 72 h then decreased towards the end of the experiment (96 h). While in temperature-controlled and stirred conditions (100 L and 22 L), the viable cell counts decreased during the first 48 h and then gradually increased and became suitable by the end of the experiment; the spore count, however, remained suitable for the first 48 h and after that it started increasing until the end of experiment. The viable cells incremented to 1.9-fold, 0.8-fold and 1.2-fold respectively for 10 L, 22 L and 100 L. The spore count incremented values were 171.6, 1.9 and 3.8 for 10 L, 22 L and 100 L, respectively. The increase in viable cell counts and fast spore generation in non-temperature controlled conditions (10 L) compared to 22 L and 100 L can be attributed to the thermophilic conditions or lack of nutrients or stress in the solid matrix [127] (Tableau.2-4). In another study, *Bt kurstaki* was used as inoculum for the fermentation of primary and secondary sludge. The lower entomoxicity was observed at high sludge concentrations due to oxygen transfer limitations [192]. Sludge subjected to alkaline PT, which increased the accessible nutrient content, which in turn increased the growth of Bt and entomoxicity [148]. After fermentation followed by alkaline PT, the sporulation rate for sludge was in the range of 38-47%; however, it was lower than the industrial TSB medium in which it was 65%. The lower sporulation rate was obtained due to the presence of heavy metals and reduced oxygen concentration in the treated sludge. The lowest LC_{50} obtained during Bt sporulation was similar to TSB and showed its highest activity against *Lepidoptera*. Moreover, TS concentration, and oxygen availability also play major roles in bacterial growth [148] (Tableau 2.4).

2.8 FUTURE PROSPECTS & CONCLUSION

The industrial sludge treatment chemicals sector was valued at about US$4.5 billion in 2016 and is expected to reach US$7.5 billion by 2024 at a CAGR of about 6%. The production rate of sludge is concerning environmentalists across the globe. But this nutrient and microbial rich matter can be viewed as a source of resources instead of as waste, which has made wastewater treatment experts consider the recovery of VAP from sludge. Increasing pressure from society, stringent disposal, an ever-increasing demand and rapid depletion of non-renewable resources

are the major factors behind this consensus. However, the presence of organic and inorganic contaminants, as well as pathogens, has impeded the use of sludge for resource recovery.

Sludge disposal techniques, such as land filling and incineration, require separate operational facilities and storage space and their associated costs are very high. In addition, the presence of toxic contaminants affects soil vegetation and further contaminates the groundwater, which, in turn, adversely affects the ecological system. Moreover, stringent disposal and societal concerns have made these disposal techniques relatively unacceptable for environmental application. Stabilization techniques, such as anaerobic/aerobic digestion, have been employed. These are eco-friendly and operationally feasible methods, which can significantly reduce the toxic content of sludge and are generally preferrable before disposal. But the hydrolysis rate remains the major obstacle preventing sludge stabilization.

To overcome these limitations, physical and chemical PT and their combinations could be applied prior to the biological process. Various physical PT techniques, such as ultrasonication, thermal, microwave, hydro cavitation, and others, were studied, and each PT was found to be successful under different applied circumstances. Out of the various PT techniques, ultrasonication and thermal treatment were preferred mostly because of low energy consumption and feasible operational conditions. While, in terms of chemical PT, the success rate was high for NaOH and ozonation related studies. These PT were effective in terms of sludge solubilization when applied separately, but in terms of VAP recovery, they were limited and less effective in contaminant removal.

On the other hand, PT assisted biological processes were found to be effective for sludge stabilization and VAP production. Furthermore, the addition of new bacterial formulations to PT assisted biologically processed sludge further enhanced sludge stabilization and helped in the production of numerous VAPs. The need to manage sludge would rapidly increase the demands for wastewater treatment procedures by global industries, generating large amounts of sludge. Thus, sludge as a recovery source is a good alternative for its management while complying with legislative requirements and circular economy principles. Three major parameters, sludge valorization, contaminant removal and VAP type, need to be considered before converting waste

into an energy resource. However, only limited research has focused on all three parameters. Hence, more attention is required on all three of these parameters to obtain a highly economic, pollutant-free, odourless, and moreover socially acceptable resource in greater demand.

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CHAPITRE 3

MATERIELS ET METHODES

Materials and methods

3.1 SAMPLING OF BIOSOLIDS AND WWTP EFFLUENT

The dewatered biosolids (collected after belt filter press) and WWTP effluent samples were obtained from local municipal WWTP (flow rate≈12000m^3/day) in the province of Quebec, Canada. The illustration of the sludge and wastewater treatment line of the WWTP is shown below in Figure 3.1. After samples were received at the laboratory, the biosolids and effluent were stored at -20ºC and 4ºC respectively.

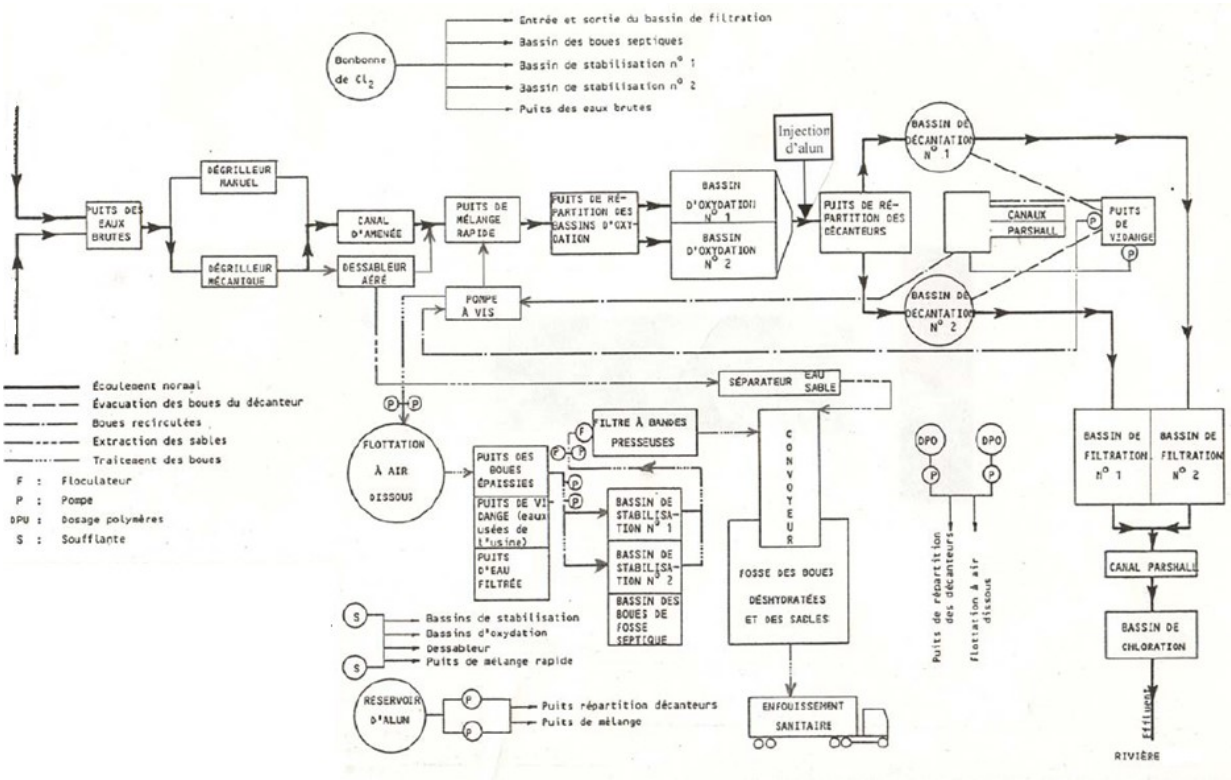

Figure 3.1- Wastewater and sludge treatment line of municipal WWTP

3.2 PREPARATION AND INITIAL CHARACTERIZATION OF BIOSOLIDS SLURRY

Biosolids stored at -20ºC after sampling was slowly thawed at 37 ºC for 12 hrs, then water generated because of freeze-thawing effect were decanted, then biosolids was mixed with effluent to prepare desired concentrations of biosolids slurry (preparation was detailed in Chapter 4,5 and 6). Prepared biosolids slurry (BS slurry) was monitored for various physicochemical properties, volumetric parameters, enzymes activities, quantification of heavy metals and TrOCs (such as pharmaceuticals and pesticides). Detailed illustration of preparation and characterization of BS slurry in Figure 3.2.

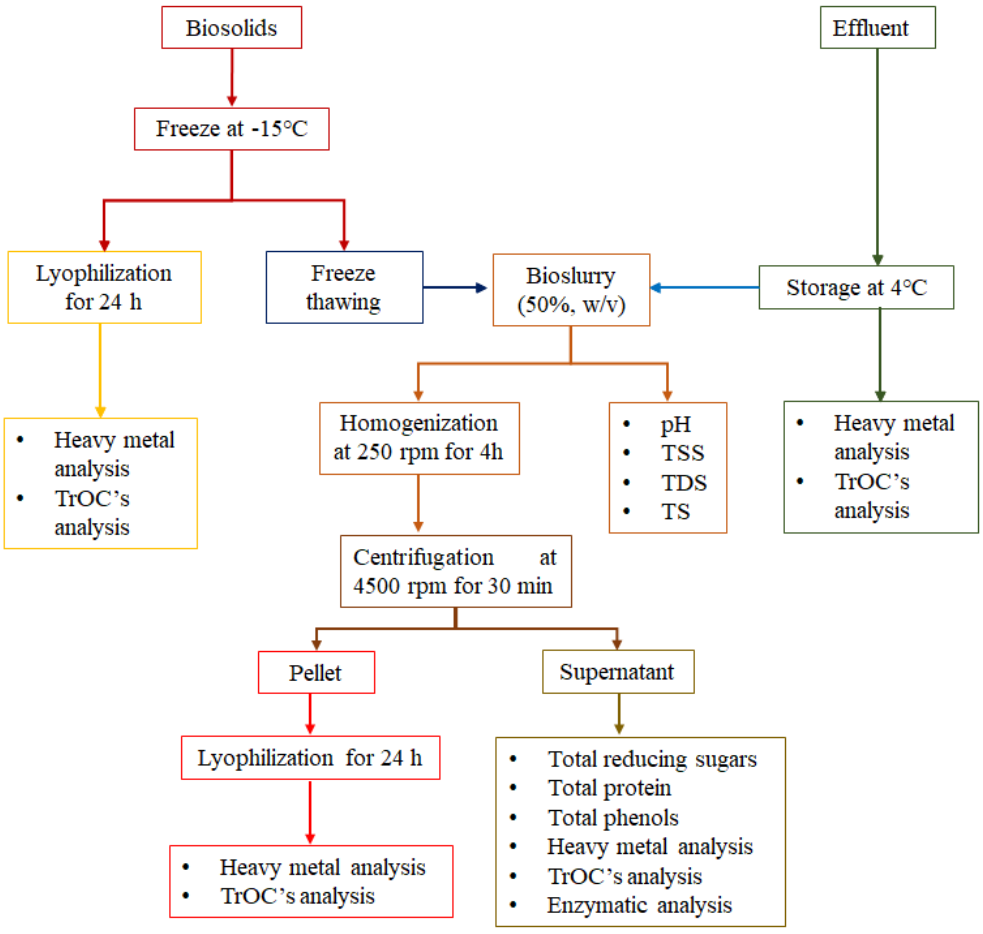

Figure 3.2- Schematic diagram of preparation and characterization of BS slurry

3.2.1 Physicochemical characterization of BS slurry

Total protein contents in the biosolids slurry was determined using bicinchoninic acid method [25]. Briefly, in a 15 mL test tube, 0.1 mL of the sample is mixed with 2 mL of BCA working reagent, this mixture is vortexed and incubated at 60˚C for 15 minutes and OD was measured at 562 nm in spectrophotometer (SpectraMax Plus 3250, Molecular Devices Corp., Sunnyvale, CA) after the samples were cooled.

Total carbohydrate contents in the biosolids slurry was determined using phenol-sulfuric acid method, where D-glucose as a standard substance [195]. To 0.5 mL of the sample, add 0.5 mL of the phenol solution and 2.5 ml of concentrated sulfuric acid. The mixture was then incubated at 90˚C for 5 minutes. After cooling of the samples, OD was measured at 490 nm in spectrophotometer.

The chemical oxygen demand (COD) was measured with the LR HACH kit [0-1500 mg / L] according to US EPA (method 8000) [25], Total nitrogen content (Method 10071) and Total phosphorus content (Method 8190) was determined by the Hach process [196].

3.2.2 Volumetric measurement of BS slurry

The biosolids volumetric parameters, total solids, total suspended solids, volatile suspended solids and total dissolved solids were measured according to the standard analysis methods [197].

Total Solids (TS)

TS was determined following the Standard Method 2540 B. The sample (1 mL of mixture in aluminum weighing dish) was evaporated to constant weight in an oven at a temperature at 105˚C for 60 minutes. The calculation is made using the equation (1):

$$mg\ total\ solids/L = (A - B)/Sample\ volume\ (mL) * 1000 \quad (1)$$

Where, A: Weight of the cup + dry residue (mg) and B: Weight of the cup (mg)

Total dissolved Solids (TDS)

TDS was determined following the Standard Method 2540 C. The sample (1 mL of mixture was filtered with standard glass fiber filter and the filtrate was evaporated to dryness in aluminum weighing dish) and dried to constant weight in an oven at a temperature at 180˚C for 60 minutes. The calculation is made using the equation (2):

$$mg\ total\ dissolved\ solids/L = (A - B)/Sample\ volume\ (mL) * 1000 \quad (2)$$

Where, A: Weight of the cup + dry residue (mg) and B: weight of the cup (mg)

Total suspended Solids (TSS)

TSS were determined following the standard method 2540 D. The sample (1 mL of mixture was filtered with standard glass fiber filter and the residue retained in the filter was evaporated to dryness) and dried to constant weight in an oven at a temperature at 105˚C for 60 minutes. The calculation is made using the equation (3).

$$mg\ total\ suspended\ solids/L = (A - B)/Sample\ volume\ (mL) * 1000 \quad (3)$$

Where, A: Weight of the filter + dry residue (mg) and B: weight of the filter (mg)

Volatile suspended Solids (VSS)

VSS were determined following the standard method 2540 E. The residue from standard method 2540 D was ignited to constant weight at 550˚C for 60 minutes. The calculation is made using the equation (4).

$$mg\ volatile\ suspended\ solids/L = (A - B)/Sample\ volume\ (mL) * 1000 \quad (4)$$

Where, A: Weight of the residue + filter before ignition (mg) and B: Weight of the residue + filter after ignition (mg)

3.2.3 Enzyme activity measurements in BS slurry

Laccase activity was measured by mixing 50 µL of the sample to 450 µL of 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as the substrate along with 500 µL 0.1M sodium acetate buffer (pH 4.0). The increase in the absorbance of the green-colored oxidized product was measured spectrophotometrically for 1 min at 420 nm [198].

Lignin peroxidase (Lip) activity was assayed by the oxidation of 90 µL sample to 105 µL of 2 mM veratryl alcohol in the presence of 300 µL of 0.1M tartrate buffer (pH 3.0) and 105 µL of 0.4 mM H_2O_2 to generate veratraldehyde, which is measured spectrophotometrically at 310 nm [198].

Aryl alcohol oxidase (AAO) activity was measured by adding 55 µL of the sample and 150 µL of 2 mM veratryl alcohol in 345 µL of 0.1M phosphate buffer (pH 6.0), followed by measuring the absorbance spectrophotometrically at 310 nm [198].

Protease activity was measured using thermally equilibrated 0.65% (m/v) casein substrate and 5 ml of 110 mM trichloroacetic acid. After incubating the samples at 37℃ for 30 min, 5 ml of 500 mM sodium carbonate solution was added to filtered samples, followed by the addition of 1 ml of Folin's reagent. Then, after incubating the samples at 37°C for 30 min, the absorbance was measured at 660 nm [199].

Phosphatase activity was determined by adding 0.5 ml of 0.115 M paranitrophenyl phosphate and 2.0 mL of 0.1 M pH 6.5 maleate buffer to 1 ml of sample, followed by incubation at 37°C for 90 min. After cooling, 0.5 ml of 0.5 M calcium chloride and 2 ml of 0.5 M sodium hydroxide was added and then centrifuged at 2580 x g for 5 min. The absorbance of the formed para-nitrophenol was measured at 398 nm [200,201].

α-amylase activity was determined by mixing 0.25 ml of 0.2% (w/v) soluble starch dissolved in 0.1 M phosphate buffer (pH 6.5) and then 0.25 ml of supernatant was incubated at 60° C for 10 min. Next, add 0.5 ml of DNS reagent and boil the solution for 5 minutes. After heating dilute the sample to 3 ml with distilled water. Read the absorbance at 540nm [57].

Lipase activity was determined by mixing 100 µL of sample to 100 µL of the 50 mM paranitrophenyl phosphate as a substrate and 800µL 0.1 M pH 8 phosphate buffer. After incubation for 40°C for 5 min, then add 3 ml of 0.5N sodium carbonate solution. The absorbance of the hydrolysis of paranitrophenyl phosphate to 4-nitrophenol was monitored spectrophotometrically at 410 nm [202].

Glucose oxidase (GOx) activity was measured by mixing 0.05 ml of sample with 2 ml of 1 M glucose and 1 ml of 0.1% (w/v) p-benzoquinone in 0.9 ml of 50 mM sodium acetate buffer at pH 5.0 .The absorbance was spectrophotometrically monitored at 290 nm

Calculation of these enzyme activities were based on the molar absorption coefficient ($\varepsilon_{(ABTS+)} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$, $\varepsilon_{(veratraldehyde)} = 9300\ M^{-1}\ cm^{-1}$, $\varepsilon_{(pNPP)} = 18 \times 10^3\ M^{-1}\ cm^{-1}$, $\varepsilon_{(benzoquinone)} = 2.31\ mM^{-1}\ cm^{-1}$).One unit is defined as the amount of enzyme required for the oxidation of 1 µmol of respective substrate per minute. The illustration of various enzyme assays were defined in Figure 3.3.

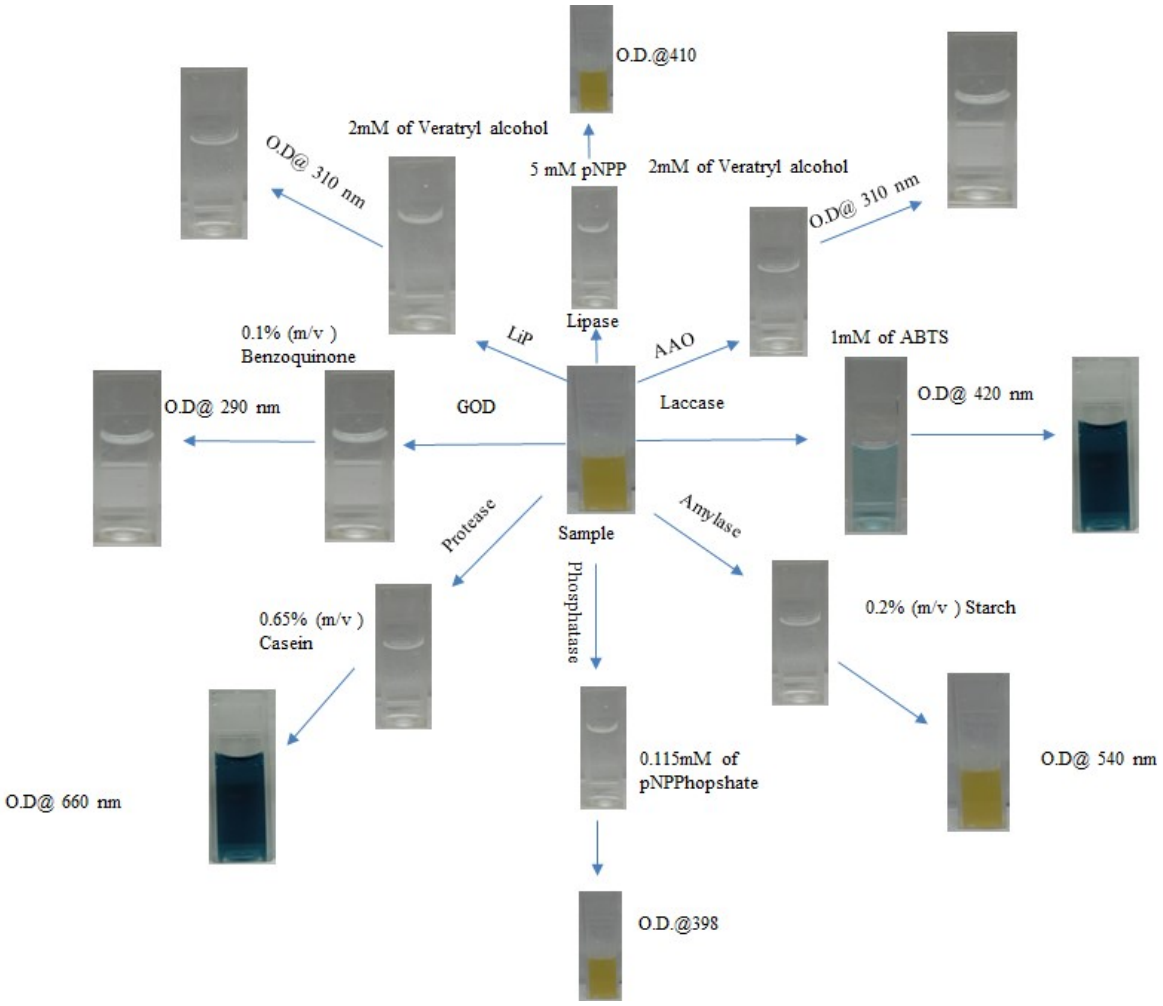

Figure 3.3- Various enzyme assays measured in BS slurry

3.2.4 Extraction and quantification of TrOCs in BS slurry

3.2.4.1 Chemicals and reagents

All chemicals, reagent used were of analytical grade, pesticides and pharmaceuticals standards, supplied in solid form (> 95% purity), were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol, Acetonitrile, Ethyl Acetate, and Formic Acid (Optima® Grade for LC / MS) were purchased from Fisher Scientific (Ottawa, ON, Canada).

3.2.4.2 Extraction of TrOCs from BS slurry

The organic contaminants (9 fungicides, 16 herbicides, 24 insecticides, and 15 PhACs), which were tested for their presence in solid and aqueous fraction of BS slurry (see Annexe 1: Tableau 4).

For solid fraction, obtained pellets were lyophilized overnight and samples were stored at -20°C until extraction. 0.25g of frozen samples were weighed and added to 4 ml of ethyl acetate (containing 1% formic acid) and 1 ml of graded water (1% formic acid) respectively. Then, the samples were vortexed vigorously and sonicated for 60s, followed by extraction by salting out with 0.4g of anhydride sodium sulphate, 0.1g of sodium chloride and 0.4g of ammonium acetate. Subsequently, the samples were vortexed and sonicated immediately for 60s to avoid bulk salt formation. Samples were then centrifuged at 4500 rpm for 20 minutes at room temperature and the upper organic layer obtained after centrifugation was removed and evaporated to dryness under a gentle N_2 stream in sand bath. The residual extracts were dissolved in 1 ml of acetonitrile (1% formic acid), then vortexed and sonicated for 60s. A purification step was carried out by mixing 0.2 g of anhydride sodium sulphate and 0.05g of C_{18} to 1 ml of crude extract. Crude extract containing salt was vortexed and sonicated for 60s followed by centrifugation at 4500 rpm for 20 minutes at room temperature.

After centrifugation, the organic layer was separated and evaporated to dryness under a N_2 stream in sand bath. The residual extract was reconstituted in 1 ml of 50%-50% methanol-water (0.2% formic acid), then filtered through a 0.22 µm PTFE filter syringe to remove the impurities. Finally, the resulting solution was carried over to UPLC-MS/MS analysis [198,203]. TrOCs extraction from solid fraction of BS slurry was illustrated in Figure 3.4.

Figure 3.4-TrOCs extraction from solid fraction of BS slurry

For the aqueous fraction, supernatant was filtered with a 0.7µm filter to remove suspended solid particles, then 1 ml of filtered supernatant was added to precondition Oasis HLB 1cc Vac cartridges with 2 ml of methanol followed by 2 ml of water. After sample addition, the cartridges were washed with 2 ml of water and eluted with 1 ml of methanol. The extracted samples were evaporated under N_2 stream in sand bath, and the residual extract was reconstituted in 1 ml of 50%-50% methanol-water and filtered with 0.22µm PTFE filter. The reconstituted sample was then carried over for analysis [198,203]. TrOCs extraction from aqueous fraction of BS slurry was illustrated in Figure 3.5.

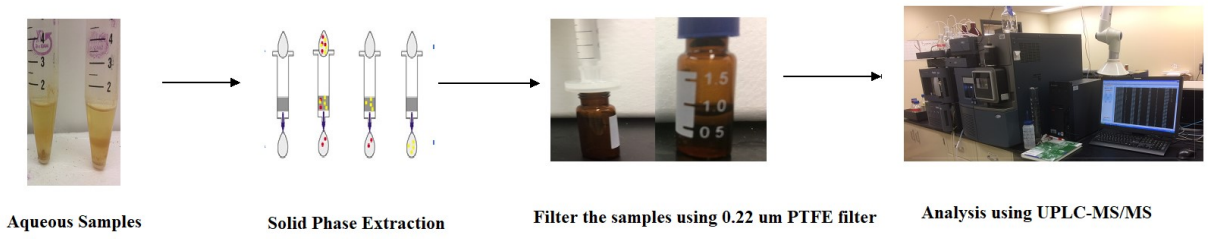

Figure 3.5-TrOCs extraction from aqueous fraction of BS slurry

3.2.4.3 TrOCs quantification using UPLC MS/MS

The quantification of the TrOCs was performed using an Acquity UPLC XEVO TQ mass spectrometer equipped with an Acquity UPLC HSS-T3 column (100 mm x 2.1 mm, 1.8 µm, with a 0.2 µm fritted pre-filter) (Waters Corporation, Milford, MA, USA) [25]. The UPLC-MS/MS analysis was performed with the HSS-T3 column in elution gradient with mobile phases of 0.2% formic acid (A) and methanol and acetonitrile (80:20, v/v) (B). The gradient was set with an initial 5% of solution B for 1 min, followed by a step increase to 80%, after 5min of hold, the gradient was increased to 90% in 1 min with a hold of 2min. The flow rate was set as 0.40 mL/min and the oven temperature of the chromatographic column set at 35 ° C. The injection volume is 5 µL. The mass spectrometry study was done in multiple reaction monitoring mode using a positive electrospray ionization source. The software Masslynx 4.1 (Waters Corporation, Milford, MA, USA) was used for MS/MS data acquisition and processing [25,203].

3.2.5 Extraction and quantification of heavy metals in BS slurry

The experiment were carried on a PerkinElmer ELAN DRC II ICP-MS, equipped with an AS-93 autosampler. The method is set as follow: 20 sweeps per reading, 1 reading per replicate and 3 replicates for each sample. The dwell time is 50 ms/AMU. Germanium is used as internal standard. Both internal standard and sample are introduced in the machine via a peristaltic pump and mixed in a T just before entering the nebulizer. The injection sequence is 45 sec of wash with 2% nitric acid, 75 sec of flush with sample, 15 sec of read delay before reading. Standard curve for calculation is prepared from 1000 ppm ICP-MS grade single element standard (Isospec, Delta Scientific) and 2% nitric acid as diluting solvent. Calibration goes from 1 to 500 ppb. Diluting solvent and wash solution are prepared from Aristar plus nitric acid (BDH) and ultrapure water (18.2 MΩ/cm, ELGA purifying machine). Samples are kept at room temperature between reception and analysis. Aqueous fraction were diluted 1mL/10 mL of 2% nitric acid. Solid fraction (100mg) are digested in 10 mL of 70% nitric acid, transferred into 100 mL volumetric flask and completed with water. This solution is diluted 1mL/10 mL of 2% nitric acid [204]

3.3 AEROBIC DIGESTION EXPERIMENTS OF BIOSOLIDS SLURRY

BS slurry were prepared with four different approaches (T1- Natural attenuation digestion, T2- Biocatalysis aided digestion, T3- Biostimulation aided digestion, T4- Biocatalysis and biostimulation aided digestion) and aerobically digested for over 28 days (Chapter 4).For detailed preparation of experiments see Tableau 4-1.

Physicochemical properties (see Section.3.2.1), volumetric parameters (see Section.3.2.2) and enzyme activities (see Section.3.2.3) were monitored on 0, 2, 4, 7, 14, 21 and 28 days of aerobic digestion. TrOCs analysis (see Section.3.2.4) were performed only on 0^{th} day and 28^{th} day of aerobic digestion.

3.4 PRETREATMENT ASSISTED BIOAUGMENTATION AIDED AEROBIC DIGESTION EXPERIMENTS OF BIOSOLIDS SLURRY

BS slurry was pretreated with four different PT (Ultrasonication, Enzymatic, Freeze-dried and Alkaline addition), then pretreated BS slurry was bioaugmented with *Bacillus subtilis* and aerobically digested for over 28 days (Chapter 5).

Physicochemical properties (see Section.3.2.1), volumetric parameters (see Section.3.2.2) and enzyme activities (see Section.3.2.3) were monitored at three different stages of the experiment. First, for raw BS slurry (no PT-0th day), then after various PT and finally during the course of bioaugmentation aided aerobic digestion (7,14,21 and 28 days) (Figure 3.6).

TrOCs analysis were performed at three different stages of the experiment (i.e; raw BS slurry (0th day), then pretreated BS slurry and finally after bioaugmentation aided aerobic digestion (only on 28th day))

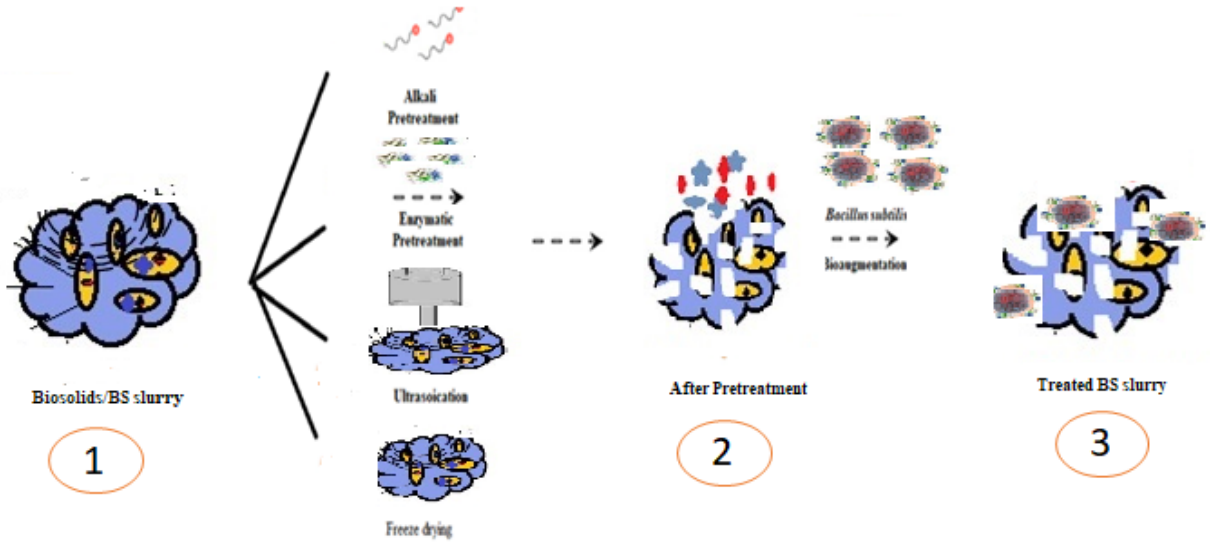

Figure 3.6- Sampling at various stages of the experiment

CHAPITRE 4

EFFET DES MICROORGANISMES INDIGÈNES ET DE LEUR ENRICHISSEMENT SUR LA VALORISATION DES BIOSOLIDES

Effect of indigenous microorganisms and their enrichment on biosolids valorization

Avant-propos

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Résumé en français : Les contaminants organiques à l'état de traces (TrOCs) dans les biosolides représentent des menaces pour la réutilisation et la valorisation des biosolides. Dans ce projet, parmi les 64 TrOCs testés, sept composés pharmaceutiques actifs (PhACs) et trois pesticides ont été détectés dans des biosolides d'une station d'épuration municipale. Cette étude comprend l'élimination des TrOCs et l'amélioration de la digestion aérobie des biosolides par divers prétraitements y compris l'utilisation de microbes indigènes présents dans les biosolides (T1), l'effet d'un prétraitement enzymatique (T2), la biostimulation des microorganismes endogènes par ajout d'une source externe de carbone (T3) et l'effet synergique de la biostimulation et du prétraitement enzymatique (T4). Après 28 jours de traitement, l'élimination des PhACs était de 44% avec T1, et de 51%, 54% et 62% avec T2, T3 et T4, respectivement. L'élimination des pesticides était de 10% avec T1, et elle est passée à 44%, 14% et 54% avec T2, T3 et T4, respectivement. Les activités de certaines enzymes extracellulaires ont également été mesurées pour tous les traitements. Les activités enzymatiques maximales obtenues (114 ± 11 U/L lipase, 382 ± 29 U/L phosphatase, 155 ± 8 U/L protéase, 304 ± 26 U/L amylase, 108 ± 7 U/L de laccase et 63 ± 2 U/L de lignine peroxydase) ont été observés pour T4 après 28 jours. Cette étude fournie des aspects changeants des profils enzymatiques au cours de ces processus et de la biorestauration améliorée des biosolides grâces aux enzymes hydrolytiques et oxydoréductases produites par les microorganismes indigènes.

Mots-clés : Biosolides municipaux, Biostimulation, Bioremédiation, Cocktail enzymatique, Tracer les contaminants organiques

Abstract: Trace organic contaminants (TrOCs) in biosolids is creating potential threats for reuse of biosolids. Out of the tested 64 trace organic contaminants, seven pharmaceutically active compounds (PhACs), and three pesticides were detected in biosolids from a municipal wastewater treatment plant. This study encompasses the removal of TrOCs and improvement in the aerobic digestion of biosolids by various PT including utilization of indigenous microbes present in biosolids (T1), the effect of an enzymatic PT (T2), biostimulation by the addition of an external carbon source (T3) and the synergic effect of biostimulation and enzymatic PT (T4). After 28 days of aerobic digestion, total PhACs removal was 44% with T1, which improved to 51%, 54% and 62% in T2, T3 and T4, respectively. Also, total pesticides removal was 10% in T1, which enhanced to 44%, 14% and 54% in T2, T3 and T4, respectively. The extracellular

enzyme activities were also monitored in all the treatments and the maximum activities (114 ± 11 U/L lipase, 382 ± 29 U/L phosphatase, 155 ± 8 U/L protease, 304 ± 26 U/L amylase, 108 ± 7 U/L laccase, and 63 ± 2 U/L lignin peroxidase) were observed in T4 after 28 days of digestion. Thus, this study aids in providing changing aspects of enzyme profiles during these processes and the enhanced bioremediation of biosolids through the hydrolytic and oxidoreductase enzymes produced by the indigenous microorganisms.

Keywords: Municipal Biosolids; Biostimulation; Bioremediation; Enzyme Cocktail; Trace Organic Contaminants

4.1 INTRODUCTION

The generation of biosolids, which are dry organic matter produced from wastewater treatment plants (WWTPs), is increasing linearly across the globe, especially in Canada. Canada produces around 6.6 million metric tons of biosolids annually [2]. Due to stringent environmental laws imposed on the disposal and application of biosolids, biosolids management has garnered much interest in recent years. Biosolids management alone could account for approximately 50% of the total operating costs of WWTPs. The disposal of biosolids by incineration or in landfills does not facilitate the recovery of valued organic matter present in biosolids and has a negative impact on the environment and human health. The strict laws and green tax levied on the incineration or landfilling of biosolids (up to $30/ton-$100/ton) in the Province of Quebec, Canada has led to extensive research on the valorization and volume minimization of biosolids [205]. However, only 51% of the total biosolids (38% as soil amendment and 13% as compost) in Quebec are actually utilized for valorization [15,206]. Recent research has documented the presence of various trace organic contaminants (TrOCs), such as pharmaceuticals, pesticides and personal care products in biosolids [13,207,208]. Thus, the quality of biosolids has raised concerns as they represent an important vehicle for the transfer of TrOCs from wastewaters to the soil and groundwater [26]. Hence, the inefficiency of the traditional WWTPs to biotransform these contaminants highlights the urgency to implement new processes to address the TrOCs present in biosolids [27].

To overcome the problems associated with disposal and land application of biosolids, stabilization of biosolids is mandatory. Several physical, chemical and biological treatments, including oxidation, carbon adsorption, precipitation, ion exchange, membrane filtration, electrochemistry, biodegradation and phytoremediation have been reported for the wastewater treatment in the last decade [209]. Although at present no single method is capable of adequate treatment of biosolids, biological treatment proves promising mostly because it is efficient, economical and reduces the environmental impact [210]. Recently, the biological stabilization of biosolids by biostimulation has gained momentum as the continuous nutrient addition shows enhanced activity of the indigenous microbial consortium, thus improving the degradation of contaminants and stabilization of biosolids [210]. The major challenge in biostimulation is choosing a stimulating agent, which is readily available to microorganisms and doesn't inhibit the rate of degradation [211]. Sugarcane bagasse [212], wheat straw pellets [132], and pine bark [121], have been taken into consideration as bio stimulating agents to decontaminate sludge.

Biostimulation processes are used to enhance bioremediation of biosolids through the production of hydrolytic enzymes by the indigenous microorganisms [59,199,213–215]. The hydrolytic enzymes produced by the indigenous microbial consortium of sludge play a major role in disrupting microbial cell structure, thereby increasing the rate of hydrolysis to release extracellular polymeric substances. They also improve the solubilization by breaking insoluble proteins, carbohydrates, lipids etc., to simpler assimilable forms [59,199,213–215]. [215] studied the effect of adding protease, amylase and their combined dosage (1:3 of protease and amylase) to sludge, which increased the solid hydrolysis to 39.7%, 54.2% and 68.2%, respectively. Similarly, [213] added cellulase, lipase, protease and its combination to achieve 30-50% TSS reduction in the sludge. While biostimulation and biocatalysis processes for biosolids remediation are commonly applied, there are limited studies on the dynamics of various hydrolytic and oxidoreductase enzyme profiles produced by the indigenous microorganisms during these processes. Hence, the aim of this research was to study the effect of adding treated water to microbe-laden biosolids and comparing the efficiency of various PT including utilization of indigenous microbes present in biosolids (T1), the effect of an enzymatic PT (T2), biostimulation by the addition of an external carbon source (T3) and the synergic effect of

biostimulation and enzymatic PT (T4) on the removal of TrOCs and improvement in the aerobic digestion of biosolids.

4.2 MATERIALS AND METHODS

4.2.1. Chemicals

All chemicals used in this study were of analytical grade (purity ≥ 95%) and purchased from Sigma Aldrich (Saint-Louis, MO, USA). Water was purified and deionized by the Milli-Q purification system (minimum resistivity 18 MX cm: Millipore, Billerica, MA, USA). The biosolids and 24-h composite effluent were obtained from a local municipal WWTP (QC, Canada). Pharmaceuticals, fungicides, herbicides and insecticide standards, supplied as solids (purity > 95%), were purchased from Sigma Aldrich (Saint-Louis, MO, USA). The enzyme cocktail (GTB2X), a mixture of protease, lipase and amylase enzymes, was provided by Nuvac Eco Science (Valcourt, QC, Canada).

4.2.2. Biosolid slurry preparation and characterization

The biosolids and WWTP effluent samples were obtained from a local municipal water treatment plant. After freeze-thawing, the unsterilized biosolids were diluted with unsterilized and filtered (0.7µm glass fiber filter) effluent, to obtain a bioslurry with a final concentration of 50% mass/volume (m/v) of biosolids [12,25]. The 50% (m/v) biosolids slurry was allowed to homogenize in a 250 ml Erlenmeyer flask by overnight agitation at 120 rpm. This slurry was further used for characterization and digestion. The samples were centrifuged at 1520 g for 10 minutes and the physical, and chemical parameters of the supernatant were determined. The dynamics of enzyme profiles during the tested biosolids treatment processes were also studied.

4.2.2.1. Physical, volumetric and nutrient parameters

The basic characterization of the biosolids slurry properties like pH and visual properties such as color was determined. The biosolids volumetric parameters, TS, VSS, and TSS were measured according to the standard analysis methods [197]. Carbohydrates and protein contents in the supernatants of biosolids slurry were determined using the phenol-sulfuric acid method and

bicinchoninic acid method, respectively [25,195]. The chemical oxygen demand (COD) was determined by the Hach process [25].

4.2.2.2. Trace organic contaminants profile

The organic contaminants (9 fungicides, 16 herbicides, 24 insecticides, and 15 PhACs), which were tested for their presence in solid and aqueous fraction of biosolids, are listed in see Sup.Info. Tableau 4. The analysis of organic contaminants in solid and aqueous fraction of biosolids slurry were extracted and estimated according to the protocol established by our group [12,25]. The quantification of the pesticides and pharmaceuticals was performed using an Acquity UPLC XEVO TQ mass spectrometer equipped with an Acquity UPLC HSS-T3 column (100 mm x 2.1 mm, 1.8 µm, with a 0.2 µm fritted pre-filter) (Waters Corporation, Milford, MA, USA) [25]. The UPLC-MS/MS analysis was performed with the HSS-T3 column in elution gradient with mobile phases of 0.2% formic acid (A) and methanol and acetonitrile (80:20, v/v) (B). The total run time was 12 min after an equilibration time of 3 min.

4.2.3. Enzyme activity measurements

Laccase activity was measured using 450 µL of 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as the substrate in 500 µL 0.1M sodium acetate buffer (pH 4.0). The increase in the absorbance of the green-colored oxidized product was measured spectrophotometrically for 1 min at 420 nm [198]. Lignin peroxidase activity was assayed by the oxidation of 105 µL of 2 mM veratryl alcohol in the presence of 300 µL of 0.1M tartrate buffer (pH 3.0) and 105 µL of 0.4 mM H_2O_2 to generate veratraldehyde, which is measured spectrophotometrically at 310 nm [198]. Aryl alcohol oxidase activity was measured by adding 150 µL of 2 mM veratryl alcohol in 345 µL of 0.1 M phosphate buffer (pH 6.0), followed by measuring the absorbance spectrophotometrically at 310 nm [198]. Protease activity was measured using thermally equilibrated 0.65% (m/v) casein substrate and 5 ml of 110 mM trichloroacetic acid. After incubating the samples at 37°C for 30 min, 5 ml of 500 mM sodium carbonate solution was added to filtered samples, followed by the addition of 1 ml of Folin's reagent. Then, after incubating the samples at 37°C for 30 min, the absorbance was measured at 660 nm [199]. Phosphatase activity was determined by adding 0.5 ml of 0.115 M paranitrophenyl phosphate and 2.0 mL of 0.1 M pH 6.5 maleate buffer to 1 ml of sample, followed by incubation at 37°C

for 90 min. After cooling, 0.5 ml of 0.5 M calcium chloride and 2 ml of 0.5 M sodium hydroxide was added and then centrifuged at 2580 x g for 5 min. The absorbance of the formed para-nitrophenol was measured at 398 nm [200,201]. α-amylase activity was determined by mixing 0.25 ml of 0.2% (w/v) soluble starch dissolved in 0.1 M phosphate buffer (pH 6.5) and then 0.25 ml of supernatant was incubated at 60° C for 10 min. The residual starch was determined according to [57]. Lipase activity was determined by observing the hydrolysis of paranitrophenyl phosphate to 4-nitrophenol spectrophotometrically at 410 nm [202].

4.2.4. Biosolids treatment

Prior to incubation, the prepared 50% (w/v) biosolids slurry (see section 4.2.2) was aerated for 15 min. Afterwards, a 100 ml aliquot of biosolids slurry was enriched with enzymes and/or glucose composition according to the experiment setup (Tableau 4.1). The 250 ml Erlenmeyer flasks (in triplicate) containing the biosolids slurry were incubated at 27°C, with periodic agitation (once a day) for 28 days. Sampling was performed after 2, 4, 7, 10, 14, 21 and 28 days of incubation. All the samples were measured for TS, TSS, VSS, soluble proteins, soluble carbohydrates, and enzyme activities. The efficiency of the tested remediation strategies to reduce PhACs, fungicides, herbicides and insecticides in solid and aqueous fraction of the slurry were evaluated by comparing their initial and final concentrations.

Tableau 4.1- Experimental setup

Treatment	**Wastewater, mL**	**Biosolids, g**	**Enzyme mixture, g**	**Glucose, g**
T1 - Natural attenuation digestion	100	50	-	-
T2 - Biocatalysis aided digestion	100	50	1	-
T3 - Biostimulation aided digestion	100	50	-	5
T4 - Biocatalysis and biostimulation aided digestion	100	50	1	5

4.3 RESULTS AND DISCUSSION

4.3.1. Aerobic digestion of biosolids

Biosolids are usually comprised of 45-70% organic matter with 3-8% nitrogen and ≤ 4% calcium, sulphur, phosphorus, magnesium and potassium, which can serve as an extensive source of nutrients for both plants and microorganisms [33]. In this context, the concentrations of carbohydrates, proteins, nitrogen, and phosphorus in the 50% biosolids slurry were estimated and the mean carbohydrate concentration was 0.12±0.02 mg/ml and the mean protein concentration was 0.50±0.03 mg/ml (Tableau 4.2). These results proved the efficiency of the biosolids to supply the essential nutrients to the microbes since these are easily assimilable forms of nutrients. Further, the COD (11.3±0.7 mg/ml) reflected the amount of organic matter potentially available for oxidation, including biological oxidation. Apart from this, total phosphorus, nitrogen and carbon content along (Tableau 4.2) with the presence of necessary metal ions in the biosolids provided the necessary platform to support the growth of microbes [216].

Tableau 4.2- Characterization of biosolids

Parameter	Concentration
Total Nitrogen (mg/g)	45.2 ± 0.1
Total Carbon (mg/g)	298.3 ± 3.0
Total Hydrogen (mg/g)	47.6 ± 0.8
Total Phosphorus (mg/g)	2.13
COD (mg/ml)	11.3± 0.7
TSS (mg/ml)	70.7 ± 1.4
VSS (mg/ml)	42.9 ± 0.9
C: N ratio	6.6

Out of the 15 investigated PhACs (see Annexe 1: Tableau 4), 7 PhACs (total concentration of 46.8±11.1 µg/kg) were detected in the solid fraction of biosolids slurry (Tableau 4.3), namely caffeine (12.2±2.55 µg/kg), carbamazepine (8.40±2.26 µg/kg), fenofibrate (7.40±0.85 µg/kg), mefenamic acid (2.6±0.85 µg/kg), naproxen (4.8±1.79 µg/kg), ketoprofen (4.0±1.46 µg/kg) and trimethoprim (7.4±1.41 µg/kg). In the aqueous fraction of biosolids slurry, 4 PhACs (total concentration of 9.5±1.1 µg/L), namely caffeine (3.3±2.25 µg/L), trimethoprim (3.35±0.63 µg/L), carbamazepine (1.2±0.56 µg/L) and fenofibrate (1.65±1.06 µg/L) were detected. Out of the 9 fungicides, 16 herbicides and 24 insecticides analyzed in the initial biosolids slurry (see Annexe 1: Tableau 4) , two fungicides (total concentration of 113±33.5 µg/kg) and one herbicide (7.2±1.7 µg/kg) were detected in the solid fraction of biosolids slurry (Tableau 4.3). In the solid fraction, carbendazim (105±22.6 µg/kg), thiabendazole (8±1.1 µg/kg) and diuron (7.20±1.70 µg/kg) were observed, while in the aqueous fraction, lower concentrations of carbendazim and diuron were observed (Tableau 4.3). Even in low concentrations, these TrOCs in the biosolids slurry could pose a major threat to the environment by increasing the probability of release of these compounds to the environment through land application [198].

Tableau 4.3- Quantitative estimation of trace organic contaminants present in the biosolids

Trace organic contaminants		**Solid phase of biosolids slurry (µg/Kg)**	**Aqueous phase of biosolids slurry (µg/L)**
Pesticides	Carbendazim	105±22.6	3.7±1.16
	Thiabendazole	8.0±1.1	N.D
	Diuron	7.2±1.7	0.3±0.1
Pharmaceuticals	Naproxen	4.8±1.8	3.4±0.6
	Caffeine	12.2±2.6	3.3±2.3
	Carbamazepine	8.4±2.3	1.2±0.6
	Mefenamic acid	2.6±0.9	N.D
	Ketoprofen	4.0±1.5	N.D
	Trimethoprim	7.4±1.4	N.D
	Fenofibrate	7.4±0.9	1.7±1.1

The use of aerobic digestion has been extensively investigated on municipal biosolids from a full-scale WWTP, for the removal of pollutants, either individually or cumulatively, which is reflected in previous studies [217–219]. Enzyme activity profiles and simultaneous removal of PhACs, fungicides, and herbicides observed during the digestion of biosolids slurry by various treatment with respect to time over a period of 28 days are presented in (Figure 4.1- 4.3).

4.3.1.1 Treatment 1 - Aerobic digestion of biosolids by indigenous microbes

The protease activities showed an almost increasing trend until day 28 when the maximum activity of 49±4 U/L was obtained (Figure 4.1(a)). Phosphatase is an important enzymatic parameter that must be monitored for improving agriculture valorization of treated biosolids. Since the latter can be extensively applied as a fertilizer, the quantification of phosphatase enzyme mainly determines the availability of phosphorus to the plants. Like protease, phosphatase activity also increased until day 28 and reached a maximum of 164.7±29.5 U/L by the end of the treatment process (Figure 4.1(b)). The common microbes present in biosolids have been shown to increase phosphatase activity in biosolids applied soil [220]. Apart from preventing phosphorus runoff, phosphatase also plays a major role in sequestration of heavy metals in soil [221].

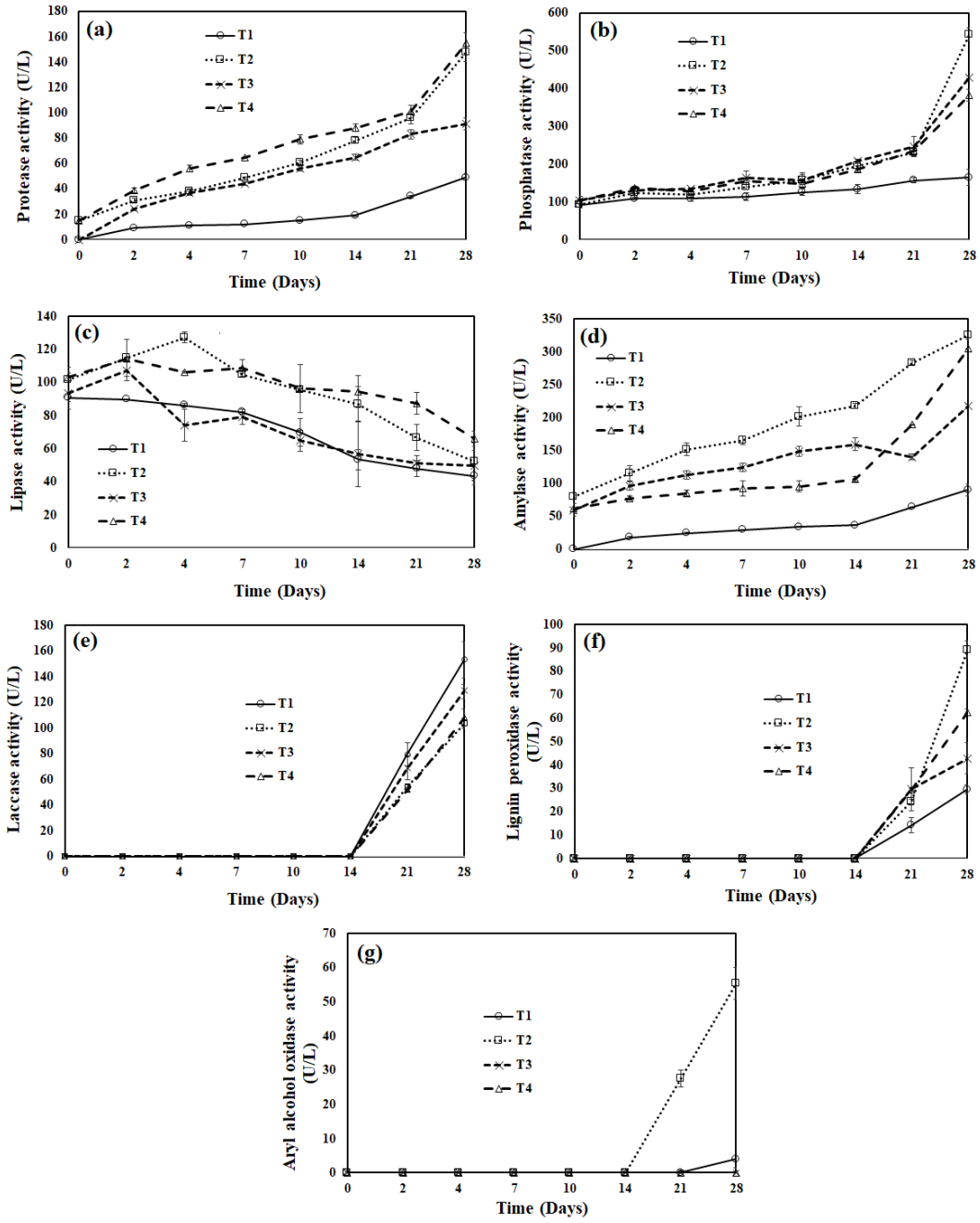

Figure 4.1- Enzyme activities observed during the digestion of biosolids slurry by various treatments (T1 – T4). (a) protease, (b) phosphatase, (c) lipase, (d) amylase, (e) laccase, (f) lignin peroxidase, and (g) aryl alcohol oxidase.

Since biosolids are a complex mixture of carbohydrates, proteins and lipids, the lignolytic enzymatic activities, amylase and lipase were also monitored. Maximum lipase activity of around 90.6±4.1 U/L was obtained at day 0 and it decreased gradually to reach minimal activity

at the end of the experiment (Figure 4.1(c)). Amylase activity was detected at day 7 and increased until day 28 to attain a maximum activity of 89.6±11.2 U/L (Figure 4.1(d)). Laccase, aryl alcohol oxidase and lignin peroxidase activities were not observed until day 14, after which an increasing trend was observed yielding a maximum of 153.4±14.1 U/L, 4.0±0.0 U/L and 29±4.6 U/L, respectively at the end of the aerobic digestion (Figure 4.1(e)-4.1(g)). The results obtained were lower than the utilization of sterilized sludge to produce lignin modifying enzymes by inoculating white rot fungi [222]. This signifies the reduced amount of easily available substrates, such as carbohydrates and proteins, which decreased after day 21 and thereby, activated the lignolytic enzyme production by the microbes. The lignolytic enzyme cocktail of laccase, lignin peroxidase and aryl alcohol oxidase produced during the treatment could be an important factor favoring the removal of organic contaminants due to their high oxidative potential [198,223].

The concentrations of PhACs, herbicides and fungicides were assessed initially and after 28 days of treatment to check the removal of the selected TrOCs (Figure 4.2-4.3). After 28 days of treatment, the total PhACs removal in solid fraction was about 44%, in which more than 80% removal of mefenamic acid, naproxen and trimethoprim was observed (Figure 4.2(a)). Contrarily, caffeine and ketoprofen accounted for poor removal of less than 30% in the solid fraction. Whereas in the aqueous fraction, more than 80% removal was achieved only for caffeine and trimethoprim. (Figure 4.2(b)) However, mefenamic acid, ketoprofen and naproxen concentrations in the aqueous fraction, which were not detected initially, increased to 1.24 ± 0.50 µg/L, 0.32 ± 0.11 µg/L and 2.68 ± 0.33 µg/L respectively (see Annexe 1: Figure 5(b)). This increase in concentration could be due to the sorption and desorption affinity of contaminants, which is difficult to properly assess because of the complex sludge matrix. Factors such as electrostatic attraction, hydrophobic bonding, etc., determine sorption of contaminants into sludge. The detected components, which were predominantly negatively charged at pH 7.0 or pH 8.0, tend to desorb from sludge due to high electrostatic repulsion, which could be the cause for increased concentrations of the concerned components in aqueous fraction [26].

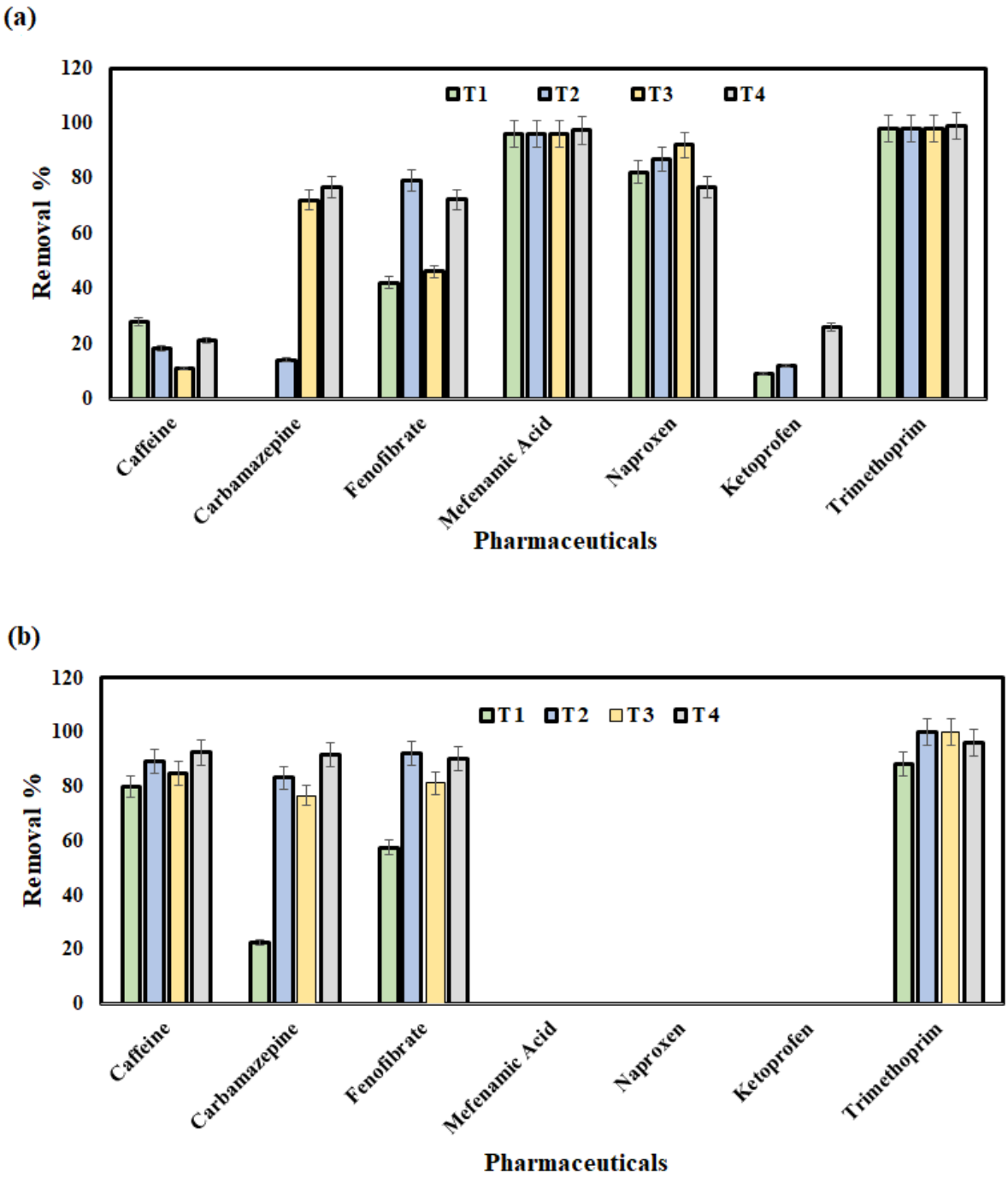

Figure 4.2- Removal of pharmaceuticals in (a) solid, and (b) aqueous phase during digestion of biosolids slurry by various treatments (T1 – T4).

T1 strategy did not perform well for the elimination of total fungicides in solid fraction, which accounted for only 5% removal from its initial concentration (Figure 4.3(a)). On the other hand, there was a pronounced herbicide removal in the solid fraction of biosolids. The detected

components, such as carbendazim, thiabendazole and diuron, were removed by approximately 4%, 14% and 92% respectively. However, the aqueous fraction indicated a good removal of carbendazim and diuron of around 92% and 98% respectively (Figure 4.3(b)).

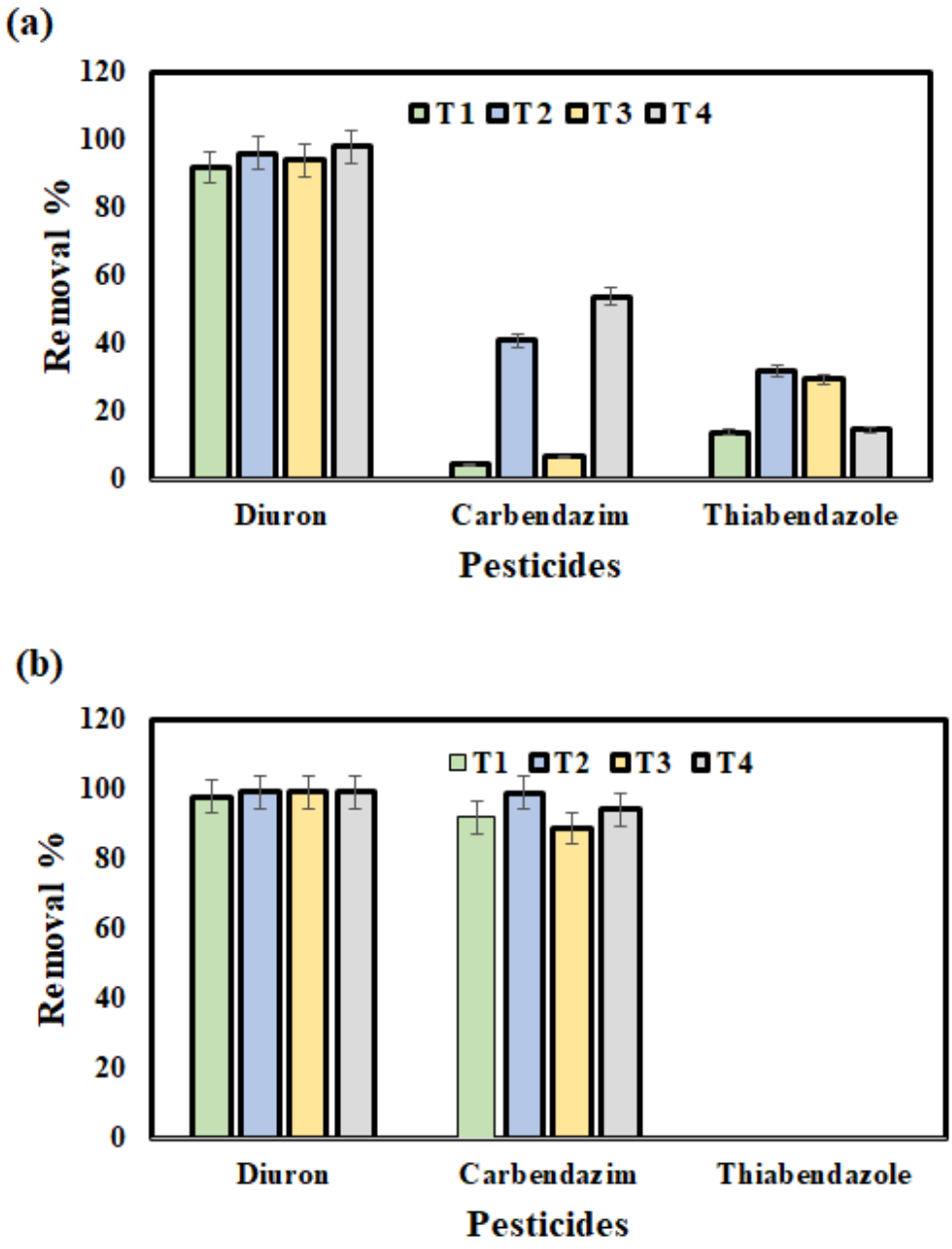

Figure 4.3- Removal of pesticides in (a) solid and (b) aqueous phase during digestion of biosolids slurry by various treatments (T1 – T4).

TSS and VSS removal during the course of treatment are shown in Figure 4.4(a) & 4.4(b). Reduction of approximately 14.5% and 18.6% for TSS and VSS removal were obtained, respectively after the treatment. The uptake of the easily soluble organic matter present in the biosolids slurry, (proteins, sugar, fatty acids, etc.,) by microorganisms probably reduced the organic load, which reflected the reduction in solid content. Moreover, enzymes such as protease, amylase, lipase and phosphatase, which play a major role in the conversion of the insoluble form of the above-mentioned molecules to soluble form, subsequently enhance the TSS removal [59]. The TSS was found to decrease and a removal of around 15.2% was obtained over a 21-day period, after which there was only a slight reduction of TSS and a removal of around 14.5% was observed at day 28. [195] reported that sludge digestion can be separated into two phases: the fast digestion phase and the stable phase. During the fast digestion phase, the rate of hydrolysis was high due to the presence of available insoluble organic matter and their easily accessible extracellular by-products and added enzymes; this was reflected by linear/fast degradation. While in the stable phase, biopolymers aggregated to form stable colloids in which the enzymes are trapped, making them inaccessible to the substrate, which affected, the hydrolysis rate resulting in decreased degradation. These findings are similar to our work, where the TSS removal was linear over 21 days, then by day 28 the removal rate stabilized. VSS reduction gradually increased to 20.5% over a 21-day period followed by a slight increase in VSS, and hence, the reduction decreased from 20.5% to 18.6% at the end of the experiment. It was observed that the reduction in VSS was synchronous with the protein and carbohydrate degradation profile. The obtained VSS removal was low when compared with work done by [57], which reported 20.9% VSS removal after 10.5 days of aerobic digestion.

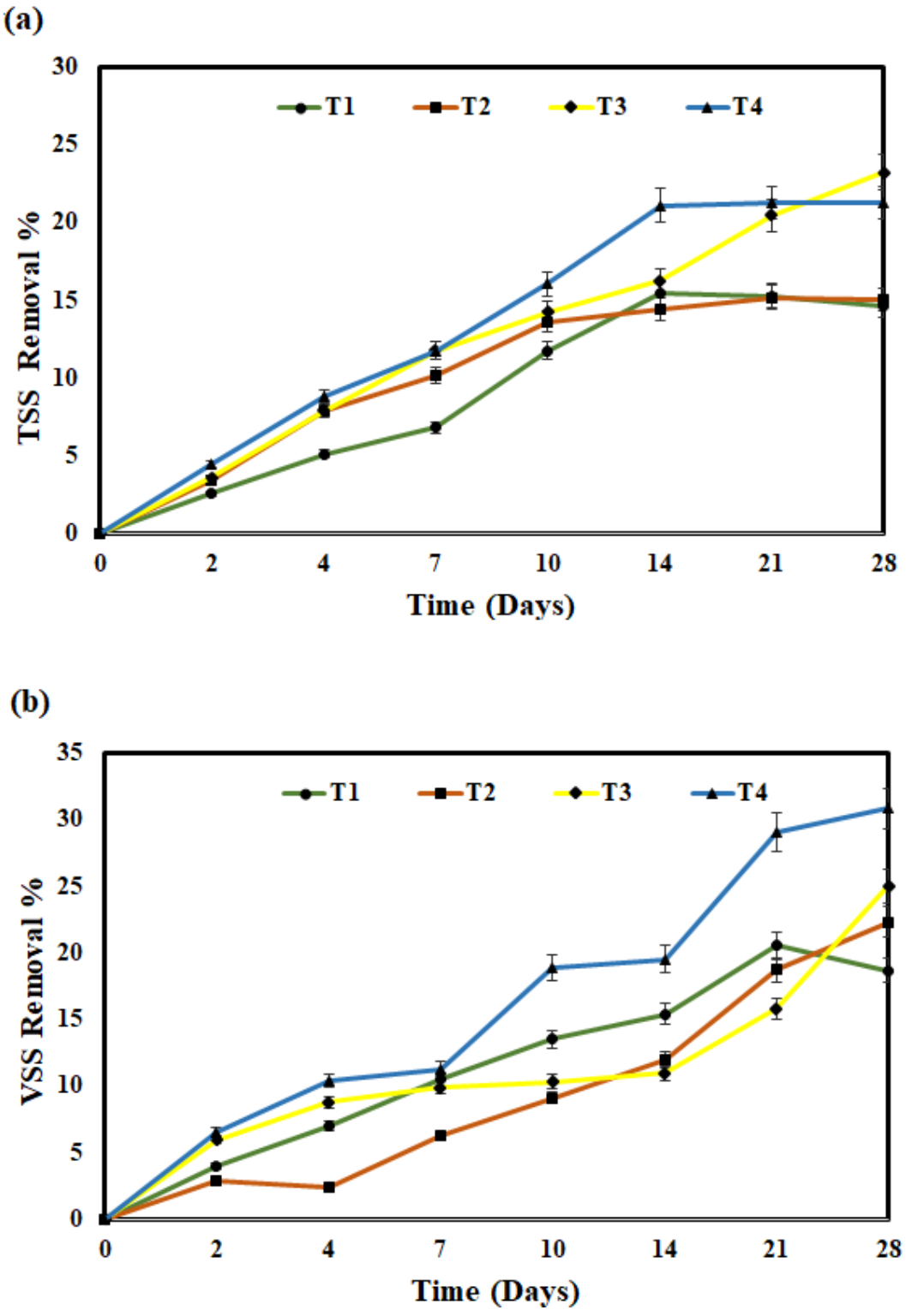

Figure 4.4- Removal of a) TSS and b) VSS during the digestion of biosolids slurry by various treatments (T1 – T4).

4.3.2 EFFECT OF PRETREATMENT ON AEROBIC DIGESTION

4.3.2.1 Treatment 2 - Effect of enzymatic PT

The increase in lipase, amylase and protease activities, which were noticed during the T2 digestion process, could be due to the increased metabolism in the indigenous microbes present in the biosolids and the wastewater used for the treatment and/or the addition of the enzymatic mixture to the biosolids slurry for the PT (T2). The phosphatase, protease and amylase activities showed a nearly linear increase until day 28 when maximum activities of 542.6±45.7 U/L, 147.2±3.2 U/L and 324.6±19.4 U/L, respectively were obtained and were 3.3, 3.0 and 3.6 times higher respectively than with T1 (Figure 4.1(a)-4.1(b) & 4.1(d)). However, similar to T1, the maximum lipase activity of 127±7.6 U/L was obtained on day 4 after which it gradually decreased and a minimal activity was obtained at the end of the treatment, (Figure 4.1(c)). It was noted that the maximum lipase activity at day 4 for T2 was 1.5 times higher than for T1 after the same incubation period. Lignolytic enzyme activities were detected after day 14, and a maximum of 103.9±11.2 U/L of laccase activity was achieved at the end of the treatment (Figure 4.1(e)). The maximum activities of the other lignolytic enzymes like lignin peroxidase and aryl alcohol oxidase were 89.2±3.9 U/L and 55.4±4.6 U/L respectively (Figure 4.1(f)-4.1(g)).

The enzymatic digestion approach (T2) removed 50.7% of total PhACs in the solid fraction of biosolids slurry after 28 days of digestion, which was merely 6.5% higher than with the T1 approach (Figure 4.2(a)). With respect to individual components in the solid fraction, the removal was similar to the T1 approach, except for carbamazepine and fenofibrate whose removal improved by 14% and 37% respectively. Whereas, in the aqueous fraction, the concentration of the total PhACs components present after 28 days of digestion was 2.5 ± 1.6µg/L, which had an initial concentration of 9.5 ± 5.3 µg/L (see Annexe 1: Figure 5b). The removal from the aqueous fraction in T2 was 47% more than T1, where all 4 initially detected components were removed by more than 80% (Figure 4.2(b)). However, the detection of naproxen, ketoprofen and mefenamic acid in the aqueous fraction was similar to that of T1. Enzymatic PT, which created favourable conditions for the microbes by not adding inhibitory components, increased the bioavailability. Furthermore, the presence of available soluble carbon

sources along with production of oxidative enzymes during digestion played a major role in the removal of contaminants from biosolids slurry.

The removal of fungicides and herbicides from the solid fraction of biosolids slurry after 28 days of digestion was around 40.3% and 96.1% respectively for T2 (Figure 4.3(a) &4.3(b)), which was 35.4% and 4% higher respectively than for T1. In addition, the removal of carbendazim and thiabendazole increased to 41% and 32%, respectively compared to 4% and 14% respectively for T1. With respect to the aqueous fraction of T2, complete removal of carbendazim and diuron was observed; however, the concentration of thiabendazole, which was not detected initially, increased to 2.12±1.01 µg/L (see Annexe 1: Figure 6(b)).

TSS and VSS reduction during T2 were around 15% and 22.3% respectively, which was 0.5% and 3.7% higher than T1 (see Annexe 1: Tableau 5). Even though the extracellular enzymes produced during the experiment were significantly higher than with other strategies, these enzymes didn't have an impact on TSS removal. This may be due to the immobilization of the enzymes produced within flocs, which made them less accessible to the substrate due to the high concentration of solids. The TSS reduction was 15.1% at day 21, and reduced to about 15.0% by day 28, thus, ensuring the stability of TSS towards the end of the experiment (see Annexe 1: Tableau 5). The protein and carbohydrate degradation profile followed a similar trend between day 21 and day 28. The results obtained were lower than in previous studies on enzymatic PT for enhancing digestion using hydrolytic enzymes, where 29-80% was reported [214] Moreover, the solid reduction achieved was low; only enzymatic treatment had been reported to yield higher solid removal in biosolids by [224]. In this study, the VSS decreased gradually from an initial concentration of 41.7 mg/ml to 32.4 mg/ml, finally reaching 22.3% removal by the end of the experiment.

4.3.2.2 Treatment 3 - Effect of glucose addition

The utilization of glucose as an external carbon source has great potential for microbial growth and enzyme production through submerged fermentation in sludge [216]. The hydrolytic enzyme cocktail present in the aqueous phase, consisting of protease, phosphatase, amylase, and lipase,

was enhanced by glucose addition. A maximum of 91.4±4.7 U/L of protease, 429.2±55.7 U/L of phosphatase and 217.5±14.1 U/L of amylase was achieved by day 28 which was 1.8, 2.6 and 2.4 times higher than the T1 samples respectively after the same duration, but 0.6, 0.8 and 0.7 times lower than the T2 samples respectively (Figure 4.1(a)-4.1(b) & 4.1(d)). The maximum lipase activity of around 107.1±6.3 U/L was obtained on day 4, which was similar to T2 (Figure.4.1(c)).

Laccase activity followed a similar trend as the untreated samples (T1) but was less active, which could possibly be due to the higher availability of carbohydrates due to glucose addition. The lignolytic enzyme cocktail yielded by this process was similar to the T1 approach and a maximum of 129±4.7 U/L laccase and 43±6.6 U/L lignin peroxidase was attained at the end of the experiment; however, no aryl alcohol oxidase activity was observed during the 28 days of treatment (Figure 4.1(e)-4.1(g)).

After 28 days of treatment by T3 strategy, total pharmaceutical removal in the solid fraction was 53.8%, which was 3% and 9% higher than T1 and T2 respectively (Figure 4.2(a)). Carbamazepine, which remained almost unaltered during T1 and T2, was removed up to 72% through the biostimulation approach (T3). Co-metabolism/metabolism is the pathway which microorganisms choose to degrade micropollutants in the ecosystem. Biodegradation occurs when the presence of contaminants along with other carbon sources, leads to the mineralization of components. On the contrary, the results obtained were not consistent with the findings of [225], which suggested that addition of supplementary carbon source by means of acetate did not improve the carbamazepine degradation. Apart from carbamazepine, the removal of all the other individual components was similar to either the T1 or T2 approach. With respect to the aqueous fraction, the removal by T3 remained similar or unaltered when compared to T2.

Fungicides and herbicides removal in solid fraction by T3 approach was 8.4% and 94% respectively, which was similar to that obtained during T1. With respect to the aqueous fraction of T3, 90% removal of carbendazim and complete removal of diuron was observed (Figure 4.3(b)); however, the concentration of thiabendazole, which was not detected initially, increased to 0.94±0.14 µg/L (see Annexe 1: Figure 6(b)).

In this treatment, 23.2% TSS removal was achieved, which was the maximum attained in these digestion experiments (Figure 4.4(a)). As a result of the addition of supplementary carbon sources, VSS reduction improved to 25% which was 6.4% and 2.7% higher than both T1 and T2 respectively. As the reduction profile is the same as that of T2, due to the inclusion of supplementary sources, the nutrient availability for microorganisms increased, which reflected the increase in the removal rate in T3. Although the glucose addition proved to be efficient in yielding better PhACs removal and solid reduction than other treatments, less TSS was removed than with ultrasonic pretreated aerobic digestion of sludge [57].

4.3.2.3 Treatment 4 - Synergistic effect of glucose addition and enzymatic pretreatment

The hydrolytic enzyme cocktail consisting of protease, phosphatase, amylase and lipase secretion was enhanced by the synergetic effect of glucose addition and enzyme PT compared to the T1 approach but there was little improvement compared to the T2 and T3 approaches. The maximum protease activity was 155±8 U/L at day 28, which was 3.2, 1.0 and 1.7 times higher than the T1, T2 and T3 approaches respectively during the same incubation time (Figure 4.1(a)). The maximum phosphatase activity was reached at day 28 and was 382.4±29 U/L, which was 2.3 times higher than with the T1 approach but 1.4 and 1.1 times lower than with the T2 and T3 approaches respectively (Figure 4.1(b)). Amylase, with a maximum activity of 304.3±26 U/L at day 28, was 3.4 and 1.4 times higher than with the T1 and T3 approaches respectively but was 0.9 times lower than with the T2 approach (Figure 4.1(d)). The maximum lipase activity of 114.1±11.3 U/L was achieved at day 2, which was 1.3 and 1 times higher than in the T1 and T3 approaches. Furthermore, similar lipase activity was observed in T4 when compared to the T2 approach for the same incubation time (Figure 4.1(c)). As with all the treatment approaches of this study the lignolytic enzyme activities were initially detected at day 14. The maximum laccase and lignin peroxidase activities, which were attained at day 28 of the treatment, were 108.1±6.9 U/L and 62.6±2 U/L respectively, which were similar to those of the individual pretreated sample (T2) activities obtained.

The T4 approach accounted for 62% of the total PhACs removal in the solid fraction of biosolids slurry, which was the maximum removal obtained during this study (Figure 4.2(a)). A complete removal of trimethoprim, mefenamic acid and approximately an 80% removal in naproxen, carbamazepine and fenofibrate was noticed. There was significant removal (83.3%) of carbamazepine unlike solar irradiation pretreated aerobic/anaerobic digestion as reported previously [217]. In the aqueous fraction of biosolids slurry, a total pharmaceutical removal of 90% was observed. Moreover, naproxen and mefenamic acid, which were detected in the aqueous fraction of the T1, T2 and T3 approaches, were not observed during the T4 approach (see Annexe 1: Figure 5(b)).

Regarding the fungicide's removal from the solid fraction of biosolids slurry, a 51.2% removal was noted, which was relatively higher than all other treatment approaches (Figure 4.3(a)). In particular, more than 50% of the carbendazim removal dictated the significance of the synergetic effect of the combined process. With respect to removal in the aqueous fraction of biosolids slurry, T4 yielded similar results to the T1 approach and carbendazim removal was greater than 94% (Figure 4.3(b)).

The TSS removal drastically increased to 21% after a 14-day period, which was approximately 5.6%, 6.6% and 4.8% higher than with the T1, T2 and T3 approaches respectively for the same period (Figure 4.4(a)). However, the removal did not improve later and remained stable at 21.2% until the end of the experiment. The addition of carbon sources and enzymatic PT enriched microorganisms enabling them to break down the soluble organics. However, upon depletion of the supplemented sources, the breakdown of these molecules was slightly affected, coupled with the inefficiency of the produced enzymes to access the substrate, which attributed to lowered TSS removal. The TSS reduction attained was slightly lower than with the T3 approach but relatively higher than the T2 and T1 approaches (Figure 4.4(a)). The VSS reduction was 30.8% which was similar to the T3 approach.

4.4 CONCLUSION

In an attempt to utilize indigenous microorganisms for solid and contaminant removal in biosolids, the following conclusions were drawn. (a) Characterization of biosolids demonstrated that biosolids are a rich source of C, N and P with the nominal carbohydrate and protein content required for microbial growth. (b) Indigenous microorganisms with the available nutrient sources (T1) were found to be ineffective against carbamazepine, mefenamic acid and naproxen, which were degraded to some extent with enzymatic PT (T2), with the addition of supplementary carbon (T3) and synergic effect (T4). (c) Even though the solid reduction achieved during this study was comparatively less than in previously reported works, removal of pharmaceuticals, herbicides and fungicides in these processes were well-pronounced. (d) Synergic effect of glucose addition and enzymatic PT prior to biosolids digestion showed the best results when compared to the other PT tested. Total PhACs, fungicide and herbicide removals of 62%, 51% and 98% respectively, were noted along with a TSS removal of 21.2%.

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CHAPITRE 5

ÉVALUATION DE L'EFFET SYNERGIQUE DE LA BIOAUGMENTATION ASSISTÉE PAR PRÉTRAITEMENT AVEC LES MICRO ORGANISMES INDIGÈNES SUR LA VALORISATION DES BIOSOLIDES

Evaluation of the synergic effect of bioaugmentation assisted by pretreatment with indigenous microorganisms on valorization of biosolids

Avant-propos

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Résumé en français : Les biosolides issus des usines de traitement des eaux usées sont un canal crucial pour le transfert des contaminants organiques à l'état de traces (TrOCs) des eaux usées vers le sol. Pour une meilleure gestion, il est nécessaire de traiter les biosolides avant leurs éliminations. La présente étude a permis d'évaluer l'effet de quatre stratégies de prétraitement sur la valorisation des biosolides et l'élimination des TrOCs lors de la bioaugmentation avec *B. subtilis*. L'analyse des biosolides a révélé la présence de huit produits pharmaceutiques actifs (concentration totale de 157,2 ± 43,4 µg / kg) et deux pesticides (concentration totale de 18,8 ± 5,8 µg / kg) sur les 72 TrOC étudiés. Après 28 jours, les résultats ont montré que lorsque les biosolides étaient augmentés avec *B. subtilis*, une élimination de 52% des TrOCs et une réduction des solides totaux en suspension (TSS) de 12%. Afin d'améliorer l'effet de la digestion aérobie des biosolides, quatre stratégies de prétraitement (traitement alcalin, l'ultrasonication, la lyophilisation et l'ajout d'enzymes hydrolytiques ont été évalués. Parmi ces stratégies, le prétraitement alcalin a montré une élimination maximale des TrOCs de 63% avec une réduction de 8% du TSS, suivie par ultrasonication, où l'élimination des TrOCs était de 32% avec une réduction de 5% du TSS. Pour ce qui est de la comparaison des effets combinés, la digestion aérobie des biosolides prétraités à l'aide d'enzymes a montré une meilleure élimination des TrOCs soit 90% et une réduction des TSS de 18%, suivie par le traitement alcalin, la lyophilisation et l'ultrasonication. Ainsi, cette étude établit la digestion aérobie assistée par *B. subtilis* comme un processus efficace pour le traitement des biosolides et explique l'impact des processus de prétraitement sur l'élimination des TrOCs et des TSS.

Mots-clés : Biosolides, *Bacillus subtilis*, Prétraitement, Contaminants organiques à état de traces, Enzymes

Abstract: Biosolids from wastewater treatment plants is a crucial channel for the transfer of trace organic contaminants (TrOCs) from wastewaters to the soil, which necessitates the treatment of the biosolids prior to any application. The present study evaluated the effect of four different pre-treatment strategies on biosolids valorization and the subsequent removal of TrOCs during bioaugmentation with *B.subtilis*. On analyzing the biosolids, out of the 72 TrOCs investigated, eight pharmaceuticals (total concentration of 157.2±47.9 mg/kg), and two pesticides (total concentration of 18.8±3.7 mg/kg) were detected. It was observed that when the biosolids was augmented with *B. subtilis* for 28 days, 52% TrOCs removal with a total

suspended solids (TSS) reduction of 12% was achieved. In order to enhance the effect of the aerobic digestion, four different pre-treatment strategies - alkali, ultrasonication, freeze-drying and enzymatic, were employed for biosolids management. Among these strategies, alkali pre-treatment showed the maximum TrOCs removal of 63% with 8% reduction in TSS, followed by ultrasonication, where the TrOCs removal was 32% with 5% TSS reduction. On comparing the combinational effect, the aerobic digestion of enzyme pre-treated biosolids showed an enhanced TrOCs removal of 90%, which also incurred TSS reduction of 18%, followed by the alkaline treated, freeze-dried and ultrasonicated biosolids. Thus, this study establishes *B. subtilis* aided aerobic digestion as an effective process for biosolids treatment and explains the impact of pre-treatment processes on TrOCs and TSS removal.

Keywords: Biosolids, *Bacillus subtilis*, Pretreatment, Trace Organic Contaminants, Enzymes.

5.1 INTRODUCTION

Municipal biosolids (BS) are abundant in organic matter and nutrients, and depending on its quality, can be used as a promising candidate for various land applications [26]. In order to protect the environment and public health, the occurrence of potential toxic elements in the BS is regulated before its disposal on farmlands from wastewater treatment plants (WWTP). Pharmaceuticals, pesticides, and other organic pollutants, which are listed under trace organic contaminants (TrOCs), are abundant in BS and are mostly recalcitrant during the wastewater treatment, which, due to its high affinity, is sorbed into BS floc by adsorption [26]. Moreover, the emerging contaminants could have the inherent ability to affect animals and humans by causing chronical disorders [198]. BS flocs is a bio aggregation of extracellular polymeric substances (EPS) and cells, where most of the extracellular enzymes produced by the indigenous microbes are immobilized [57]. The EPS in floc plays a major role in the absorption of TrOCs because of its high hydrophobic and binding property [26]. In order to enrichen BS treatment along with its reduction, disruption of EPS matrix by means of PT is an effective method to enhance the sludge degradation in a fixed duration [57]. Sludge disruption methods include mechanical, thermal, chemical and biological treatments [226]. These PT disrupt the bacterial

cell wall and the flocs, resulting in the release of the intercellular components to the aqueous fraction which are then readily available for subsequent transformations [226].

Ultrasonication is a mechanical disruption technique which known for its several merits due to effective solubilization and improved biodegradability [64]. Mechanism of BS destruction during ultrasonication was depicted in three stages, such as floc losing, cell breakage and macromolecule degradation, where destabilization or disruption of BS flocs results in release of EPS [227]. Specific energy of 2.45kJ/kg TS is optimal for the effective release of EPS with limited cell lysis [64]. This can improve the action of bacteria during aerobic digestion, subsequently improving the degradation rate of sludge. Apart from disruption, generation of highly oxidative reactive radicals by implosion of cavitation bubbles, breakdown of the environment micropollutant, which are generally recalcitrant, into simpler forms, thereby increasing their bioavailability for microbial degradation [228]. BS, which is subjected to high pH, disrupts its floc structure by decreasing its particle size, denaturation of protein, lipid saponification and disassociation of acid group in EPS, which modifies its electrostatic charge, subsequently increasing the hydrolysis rate of sludge [229]. The cell disruption is predominant over the range of pH 8.0 - 12.5, where microbial cell walls cannot handle the turgor pressure created, resulting in the release of intercellular substances [25,230]. For such high pH applications, NaOH is preferable over KOH or $Ca(OH)_2$, owing to its low cost and effective impact on sludge disintegration [231]. The cost of chemicals for alkaline treatment was around 0.234€/kg of sludge [69]. BS treatment and bioavailability are greatly improved due to the increase in biodegradability of the various compounds which are released from the microbial cell walls upon the alkaline treatment of BS [230].

The reports on the freeze drying as a treatment of sewage sludge for TrOCs removal are few, which are utilized mostly for dewatering and recovering biomass [232]. During freeze drying, surface water attaches to the sludge flocs, which weakens the surface area of sludge floc [232]. Considering this viewpoint, freeze drying can be applied as one of PT method for disruption of BS. Moreover, no studies have been conducted to evaluate the effect of freeze-drying based contaminant removal and subsequent aerobic digestion. When compared to several PT methods, the biological PT was found to be effective as it neither requires any mechanical equipment nor

causes environmental pollution; moreover, it can work in mild optimal conditions such as temperature and pH [102,229]. In biological PT, degradation of complex biodegradable matter is addressed by the addition of hydrolytic enzymes, which acts on sludge to release the nutrients in soluble form along with reduction in volatile suspended solids (VSS) [229,233]. Specifically, enzymes act upon particular substrates, which disintegrate the loosely bound sludge-substrate complex, leading to improved solubilization of sludge [234]. However, the solubilization rate is dependent on the nature of sludge matrix and an improved sludge solubilization using hydrolytic enzymes were previously reported by [102,234]. However, to the best of our knowledge, studies focusing on the removal of TrOCs during enzymatic PT followed by aerobic digestion can be rarely found.

Biological approaches such as aerobic/anaerobic are preferable over chemical treatment for the reduction of sewage sludge due to its eco-friendly nature and avoid harmful effects when it is emitted into environment [235]. Although anaerobic digestion is majorly used, aerobic digestion is considered in few treatment plants because of its enhanced nutrient removal, efficient organic pollutant removal, odor-free operation, easy control and social acceptability [236]. Aerobic digestion is greatly influenced by the enzymes secreted by the bacterial strains [120]. Involvement of microorganisms under aerobic conditions on decomposition of organic matter (OM) in sludge can be categorized into three phases. During first phase, easily degradable OM is solubilized by microorganisms by secreting enzymes for their rapid growth. Once the degradable OM has been utilized, microorganisms obtain energy by self-oxidation of cellular substances, which takes place in second phase. In final phase, intercellular substances continually oxidized [237].

Sludge which is stabilized after treatment is either landfilled or land applied because of its agricultural importance [25]. Therefore, sludge which contains biocontrol agents such as biopesticides will be an attractive option to use on land related applications. *B.subtilis*, a non-pathogenic eminent and potential biocontrol agent which possess an antimicrobial activity [238] and able to sustain under stressful conditions in sludge. Thus *Bacillus sp*. [235] has been considered to be the strain of interest during this study.

In this study, we focused on the effect of four different PT methods, namely ultrasonic, alkaline, freeze drying and enzymatic, on the removal of TrOCs and further the solid reduction in BS bioaugmented with *B.subtilis*. Various parameters like, chemical oxygen demand (COD), and the hydrolytic and oxidoreductase enzyme activities, which are considered as important factors contributing to the solid reduction and TrOCs removal during bioaugmentation under aerobic conditions, were also investigated in the present study.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

All reagents used were of analytical grade or better. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), veratryl alcohol, para-nitrophenyl phosphate, casein, para-nitrophenyl palmitate, soluble starch, pharmaceuticals and pesticide standards, supplied as solids (purity > 95%), were purchased from Sigma Aldrich (Saint-Louis, MO, USA). Water was purified and deionized by Milli-Q purification system (minimum resistivity 18 MX cm; Millipore, Billerica, MA, USA). GTB2X, a commercial dry powder product (Nuvac Eco Science, Valcourt, Qc, Canada), which is a mixture of lipase, protease, cellulase and amylase enzymes, was used for enzymatic PT.

5.2.2 Biosolids sampling and characterization

BS and wastewater effluent (WWE) were obtained from a local municipal WWTP in the province of Quebec, Canada. The BS and WWE collected were stored at -20°C and 4°C respectively and used within 7 days of sampling. The WWE obtained from the WWTP were mixed with the freeze-thawed unsterilized BS to obtain a final concentration of 50% (m/v) BS slurry. The 50% (m/v) BS slurry was prepared by the addition of 50 g of BS in 100 mL of treated WWE in a 500 mL Erlenmeyer flask. Volumetric parameters such as TS, TDS and TSS in the BS-slurry were measured according to standardized methods of analysis [25]. After measuring the volumetric parameters in BS slurry, the samples were centrifuged at 1520 g for 10 min to obtain the pellet and supernatant. To determine the available nutrients for microbes in the BS slurry, measurement of protein [12], total nitrogen, total phosphate, total carbohydrate content and COD [196], were analyzed in the supernatant. Further, the supernatant was filtered through a 0.45 μm membrane, and the filtrate was utilized to analyze soluble N and P, soluble carbohydrate

and soluble chemical oxygen demand (SCOD) [196]. The activities of hydrolytic and oxidoreductase enzymes, such as laccase [198], lignin peroxidase [198], aryl alcohol oxidase [198], cellulase [239], protease [102], phosphatase [240], lipase [202] and amylase [102], in the supernatant of BS slurry were assayed according to previously established studies. Analysis of BS slurry for the presence of TrOCs, such as pesticides and pharmaceuticals, was performed in UPLC-MS according to the already developed methodology [25].

5.2.3 Pretreatment of biosolids

For ultrasonication treatment, the BS slurry (see section 5.2.2) was subjected to ultrasonication (Digital Sonifier 450, Branson, CT, USA), with working condition of maximum output power – 400 watts and operating frequency-60 kHz for 10 min in a water-ice bath followed by further aerobic digestion [57]. For alkaline treatment, the pH of the prepared BS slurry was increased to 11.0 from the initial pH of 7.2 by the gradual addition of NaOH (5N). The samples were then kept in a magnetic stirrer at 240 rpm for 30 min to ensure uniform mixing, after which the samples were centrifuged at 1520 g for 10 minutes, henceforth the supernatant was decanted for measuring the physical, chemical parameters. Therefore, alkali pretreated BS were mixed with distilled water and its pH was adjusted to neutral (pH 7.0) using HCl (1M) and then processed for further aerobic digestion [241]. For enzymatic treatment, 1% (w/v) GTB2X enzyme mix was added to BS slurry and the samples were agitated at 120 rpm for 24 h, followed by aerobic digestion. For freeze drying experiments, 50 g of unsterilized freeze-thawed BS was subjected to lyophilization (Virtis benchtop 4K, USA), with working condition of 22 mTorr at -80°C for 24 h, after which, the freeze-dried BS was mixed with 100 ml of unsterilized WWE. After the mixture was stirred, the samples were processed for further aerobic digestion.

5.2.4 Bioaugmentation by *B. subtilis* for aerobic digestion

The pre-treated BS slurries (see section 5.2.3) were inoculated with 10% (w/v) of a commercially available strain of *B. subtilis* (Nuvac Eco Science, Valcourt, Qc, Canada) in a 500 mL Erlenmeyer flask. All the flasks were incubated at 27ºC with continuous agitation at 150 rpm for a period of 28 days and the experiments were performed in duplicates. Duplicates were

sacrificed every 7 days for characterization. After measuring the volumetric parameters in BS slurry, the samples were centrifuged, and supernatant was subjected to characterization of various parameters mentioned in the previous section (see section 5.2.2). TrOCs content in both the fractions of BS slurry were measured after 28 days of treatment. TrOCs removal efficiency from BS was calculated by difference between initial (BS) and final (Solid fraction) concentration in two different times during this study,

i) After PT

$$TrOCs_{removal}\ (Pretreatment) = \frac{TrOCs_{Initial}(BS) - TrOCs_{final}(Solid\ fraction_{after\ pretreatment})}{TrOCs_{Initial}(BS)} \quad (1)$$

ii) After bioaugmentation with *B. subtilis*

$$TrOCs_{removal}\ (Final) = \frac{TrOCs_{Initial}(BS) - TrOCs_{final}(Solid\ fraction_{after\ bioaugmentation})}{TrOCs_{Initial}(BS)} \quad (2)$$

5.3 RESULTS AND DISCUSSION

5.3.1 Impact of pretreatment methodologies on biosolids

5.3.1.1 Reflection in proteins, SCOD/COD ratio, carbohydrate, and organic matter content

During PT of BS slurry, disruption of the microbial cell walls results in increase of COD and SCOD (mg/L) in the supernatant due to the release of intracellular components and extracellular enzymes to the aqueous fraction [196,228]. Increase in SCOD directs the release of soluble organics from the colloidal sludge flocs after disruption. Thus, measuring SCOD/COD ratio gives an appropriate assumption of the amount of soluble organics which are derived from insoluble organics during the experiment [57]. The SCOD/COD ratio of raw BS slurry was 0.26 ± 0.03, and it was greatly improved in all the PTs (except enzymatic PT) (Figure 5.1), which indicated the presence of higher amounts of soluble organics in the supernatant. Due to high solids concentrations, enzymes were trapped inside the floc. This entrapping of enzymes made them less accessible to the substrate, could be the possible cause for no improvement of SCOD/COD ratio during enzymatic PT. Among the various PTs, alkali PT showed enhanced

solubilization, where the SCOD/COD ratio was 0.87 ± 0.11, compared to ultrasonication, freeze drying and enzymatic PT, where the ratios were 0.48 ± 0.06, 0.52 ± 0.11, and 0.18 ± 0.07, respectively (Figure 5.1). Similar studies was reported on ultrasonic PT of activated sludge, where the ratio increased from 0.04 to 0.20 [57], and in alkaline method, the solubilization increased by 33% [196]. In alkaline PT, at high salt concentration, the cell lysis was faster due to high turgor pressure created in the environment, which resulted in the higher release of organics, when compared to the other mechanical methods. Moreover, solubility of certain particulate organics is not pronounced in mechanical methods compared to the chemical counterpart [242]. The disruption experiments resulted in the release of intracellular compounds, which was due to disruption of cell walls, and thus, in turn increased the concentrations of soluble N, P and carbohydrates [196]. Out of the various PT methods, alkaline PT was found to be more effective in improving the soluble protein (soluble N and P) and carbohydrate content with the solubilization of 43%, 91% and 41%, respectively (Figure 5.1).

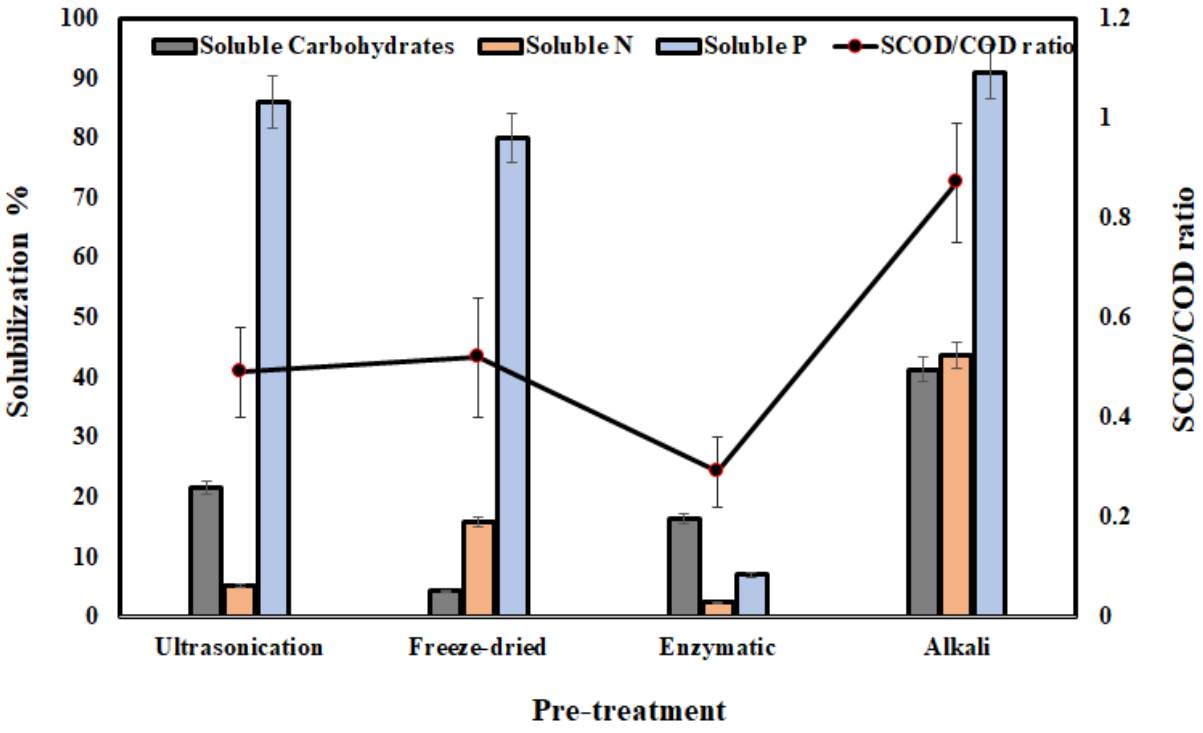

Figure 5.1- Impact of various pretreatment methodologies on nutrient parameter of biosolids. (Initial SCOD/COD ratio of 0.26±0.03, soluble N, P and Carbohydrate concentrations are 34.09 ± 0.7 mg N/L, 14.1 ± 3.9 mg P/L and 94.9 ± 15 g/L, respectively).

In ultrasonication PT, 86% and 21% solubilization was obtained for soluble P and carbohydrates, respectively. However, only 5% solubilization of N was achieved by this PT, which is an anomaly when compared to the previous studies on effectiveness of ultrasonication in solubilizing N, P and carbohydrates over alkali PT [196]. On the other hand, enzymatic PT was not much pronounced in solubilizing N, P and carbohydrates, which improved the solubilization only by 2%, 7% and 16%, respectively. This may be due to either the entrapment of the commercial enzymes in the flocs or they were inadequate to react with the available substrate. Freeze-dried PT was found to have pronounced impact on solubilizing N (15%) and P (80%), but limited impact on soluble carbohydrates, which increased by only 3%. Solubilization of carbohydrates after PTs is in the order of alkali < ultrasonication < enzymatic < freeze-dried. With respect to the concentrations of proteins, carbohydrates and SCOD/COD ratio in the aqueous fraction, it was observed that all the disruption experiments carried out had an effect on breaking the insoluble organics into soluble form, with the alkali and freeze-dried PT having a pronounced effect on the disruption process.

5.3.1.2 Effect of pretreatment on TSS content and enzymes released from biosolids

TSS removal after PT were high in alkali pretreated samples which was about 8.1% followed by 5.2% and little/negligible removal in ultrasonication and enzymatic pretreated samples, respectively (Tableau 5.1). This decrease in TSS content may be due to the conversion of insoluble organics to soluble form [57]. However, for the freeze-dried samples, the TSS content increased to 11% when compared to the TSS of untreated samples. It may be due to the complete removal of water molecules after freeze-drying, which might result in the containment of only the inactivated bacterial cells in the concentrated solid particles [232].

Tableau 5.1- Impact of various pretreatment methodologies on volumetric and nutrient parameter of biosolids

Parameter	Raw/Initial	Sonication	Freeze-dried	Enzymatic	Alkaline
Total proteins (mg/ml)	0.36 ± 0.09	0.56 ± 0.12	0.23 ± 0.02	0.40 ± 0.01	0.89 ± 0.21
Total Nitrogen (mgN/L)	35.2 ± 4.4	45.5 ± 3.2	52.2 ± 3.2	37.3 ± 4.1	61.3 ± 6.4
Soluble Nitrogen (mgN/L)	34.09 ± 0.7	35.8 ± 2.2	39.1 ± 2.6	34.8 ± 3.2	48.8 ± 4.7
Total phosphorus (mgP/L)	131.3 ± 5.7	123.1 ± 0.9	50.1 ± 2.3	142.2 ± 2.6	149.5 ± 0.6
Soluble phosphorus (mgP/L)	14.1 ± 3.9	26.2 ± 0.9	25.4 ± 0.6	15.1 ± 0.2	26.9 ± 0.0
Total Carbohydrates (mg/ml)	0.99± 0.13	1.32 ± 0.16	1.07 ± 0.13	1.21 ± 0.31	1.46± 0.29
Soluble Carbohydrates (mg/ml)	0.95 ± 0.15	1.15 ± 0.21	0.98± 0.04	1.10± 0.18	1.34 ± 0.23
COD (mg/ml)	6.9± 0.4	8.8 ± 0.9	10.9 ± 0.7	7.1 ± 0.3	16.5 ± 1.9
SCOD (mg/ml)	1.8 ± 0.2	4.3 ± 0.4	5.7 ± 1.2	1.3 ± 0.5	14.4 ± 1.0
TSS (mg/ml)	57 ± 1.2	54 ± 2.3	63. 2± 4.2	56.9 ± 0.7	52.4 ± 1.3
TDS (mg/ml)	2.4 ± 0.7	4.2 ± 1.3	3.9 ± 0.5	3.1± 0.8	13.2 ± 3.7

Various enzyme activities were detected in the raw BS slurry such as protease, lipase, amylase, cellulase and acid-phosphatase with activities of 7249 ± 112 U/L, 59 ± 3 U/L, 285 ± 29 U/L, 152 ± 17 U/L and 3226 ± 214 U/L, respectively (Figure 5.2(a) & 5.2(b)). In alkali pretreated BS slurry, except phosphatase and lipase, no other enzyme activities were observed. Due to the impact of pH triggering, the obtained enzymes were detected at a very low concentration, which

reduced approximately 5.0-fold when compared to raw BS slurry. Increased interaction between the active site and substrate during the alkali PT greatly influences enzyme activities. As a result of ultrasonication, the lipase activity increased, whereas the activities of phosphatase, cellulase and protease decreased. However, ultrasonication had no impact on amylase activity, which was inconsistent with earlier research on ultrasonication that interpreted decrease in amylase activity [57]. However, the present findings showed no inactivation of enzymes, which is comparable to previous findings [57]. Further, upon freeze-drying, the protease, amylase and phosphatase activities increased and a decrease in the lipase and cellulase activities were observed. However, during enzymatic PT, all the enzyme activities increased, which may be attributed to the addition of enzyme cocktail to the already secreted microbial enzyme present in the BS slurry. This finding can be related to previous studies on improvement of enzyme activities to 20% after 4 h incubation in enzyme treated sludge [243].

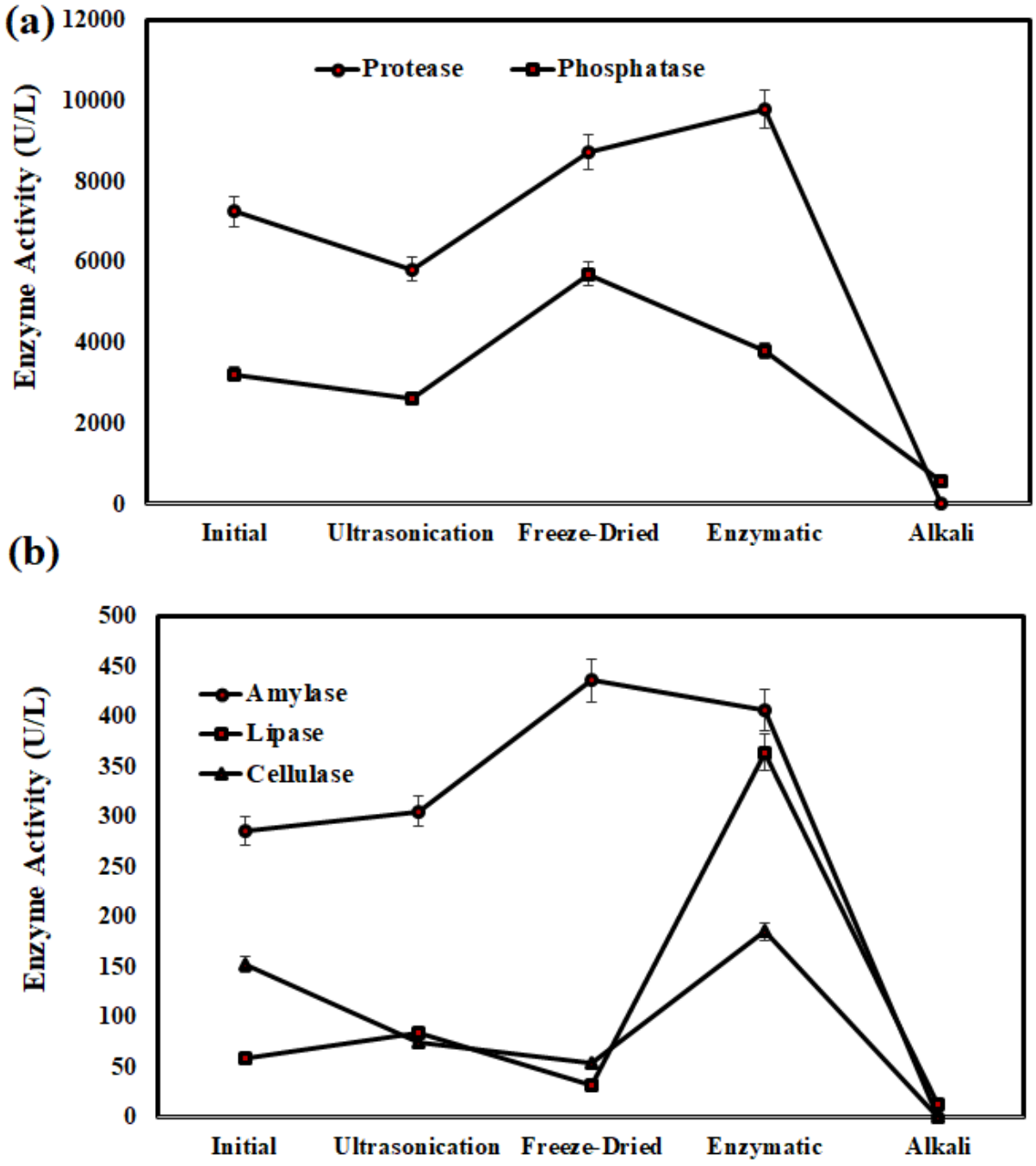

Figure 5.2- Extracellular enzyme activities (a) protease and phosphatase, (b) lipase, amylase and cellulase in control and various pre-treatment strategies on biosolids.

5.3.1.3 Effect of various pretreatments of biosolids on TrOCs concentration

Out of the 72 components analyzed, 8 pharmaceuticals (total concentration of 157.2±43.4 µg/kg) and 2 pesticides (total concentration of 18.8±5.8 µg/kg) were detected in the solid fraction of BS slurry namely, caffeine 77.2±10.8 µg/kg, acetaminophen 8.2±4.2 µg/kg, ibuprofen 25.6±12.7 µg/kg, carbamazepine 22.6±7.0 µg/kg, atenolol 2.6±0.5 µg/kg, diuron 8.8±0.8 µg/kg, atrazine 10±4.9 µg/kg, naproxen 5.6±0.5 µg/kg, cyclophosphamide 2.0±0.0 µg/kg and trimethoprim-13.4±7.6 µg/kg. Whereas, in the aqueous fraction of BS slurry, 4 pharmaceuticals (5.4±2.9 µg/L) and 1 pesticide (6.1±0.2 µg/L) namely acetaminophen 0.05±0.05 µg/L, atenolol 1.2±1.2 µg/L, carbamazepine 4±2 µg/L, trimethoprim 0.1±0.1 µg/L and atrazine 6.1±0.2 µg/L were present (Tableau 5.2).

Tableau 5.2- Initial TrOCs concentration at solid and aqueous fraction in biosolids slurry

Classes	**Pharmaceuticals/ Pesticides**	**pKa**	**Initial Concentration in biosolid slurry**	
			Solid Fraction (µg/kg)	**Aqueous fraction (µg/L)**
Anti-inflammatory and Analgesics	Acetaminophen	9.86	8.2 ± 4.2	0.1 ± 0.1
	Naproxen	4.15	5.6 ± 0.5	N.D
	Ibuprofen	4.40	25.6 ± 12.7	N.D
Antineoplastic	Cyclophosphamide	2.8	2.0 ± 0.0	N.D
Stimulant	Caffeine	10.4	77.2 ± 10.8	N.D
Psychiatric drug	Carbamazepine	14.0	22.6 ± 7.0	4.0 ± 2.0
B-blockers	Atenolol	9.67	2.6 ± 0.5	1.2 ± 1.2
Antibiotics	Trimethoprim	6.6–7.1	13.4 ± 7.6	0.1 ± 0.1
Herbicides	Diuron[a]	3.7	8.8 ± 0.8	N.D

	Atrazine[b]	1.68	10 ± 4.9	6.1 ± 0.1

*N.D- Not detected

[a] Toxic equivalent quotient (TEQ) value for diuron in solid fraction of BS slurry is 288.1 ± 27.7 µg/kg [244].

[b] TEQ value for atrazine in solid and aqueous fraction of BS slurry is 10 ± 4.9 µg/kg and 6.1 ± 0.1 µg/L respectively [244].

Disruption experiments have a pronounced impact on the removal of TrOCs from solid fraction of BS slurry. Addition of 5 M NaOH reduced the total contaminants concentration to 65.1±5.3 µg/kg from the initial of 176±49.2 µg/kg in solid fraction (see Annexe 2: Figure 7(a)). In particular, caffeine, trimethoprim, atenolol and acetaminophen have accounted for more than 70% removal (Figure 5.3). Whereas in the aqueous fraction, the concentration of acetaminophen, carbamazepine and atrazine increased to 5.0±2.9 µg/L, 7.1±0.0 µg/L and 7.35±0.05 µg/L, respectively (see Annexe 2: Fig.8). Moreover, the concentration of caffeine (pKa=10.4, neutral charge at pH 7.0) which was not detected initially in aqueous fraction, increased to 2.7±0.2 µg/L in aqueous fraction of alkali pretreated BS slurry (see Annexe 2: Figure 8). Sorptional behavior of TrOCs (ionizable) in solid fraction is subjected to alter when it possess high alkaline conditions because of dilution [26]. BS are generally negatively charged and when excess of NaOH is used for alkali treatment, Na^+ ions attach to the surface of the BS particulates, making the surface of biosolids particles saturated with Na^+ ions by forming a slimly layer and repel the TrOCs [245]. Moreover, during the treatment, some of the alkali act on the organic matter which binds to the TrOCs, thereby dissolving them, which leads to the dissociation the TrOCs [87]. On the other hand, saponification and solvation that occurs due to increase in pH, lead to the increase in COD solubilization, which attribute to desorption of extracellular polymers [87] and release of EPS, which in turn, leads to the release of TrOCs bound to EPS into to the aqueous fraction [26].

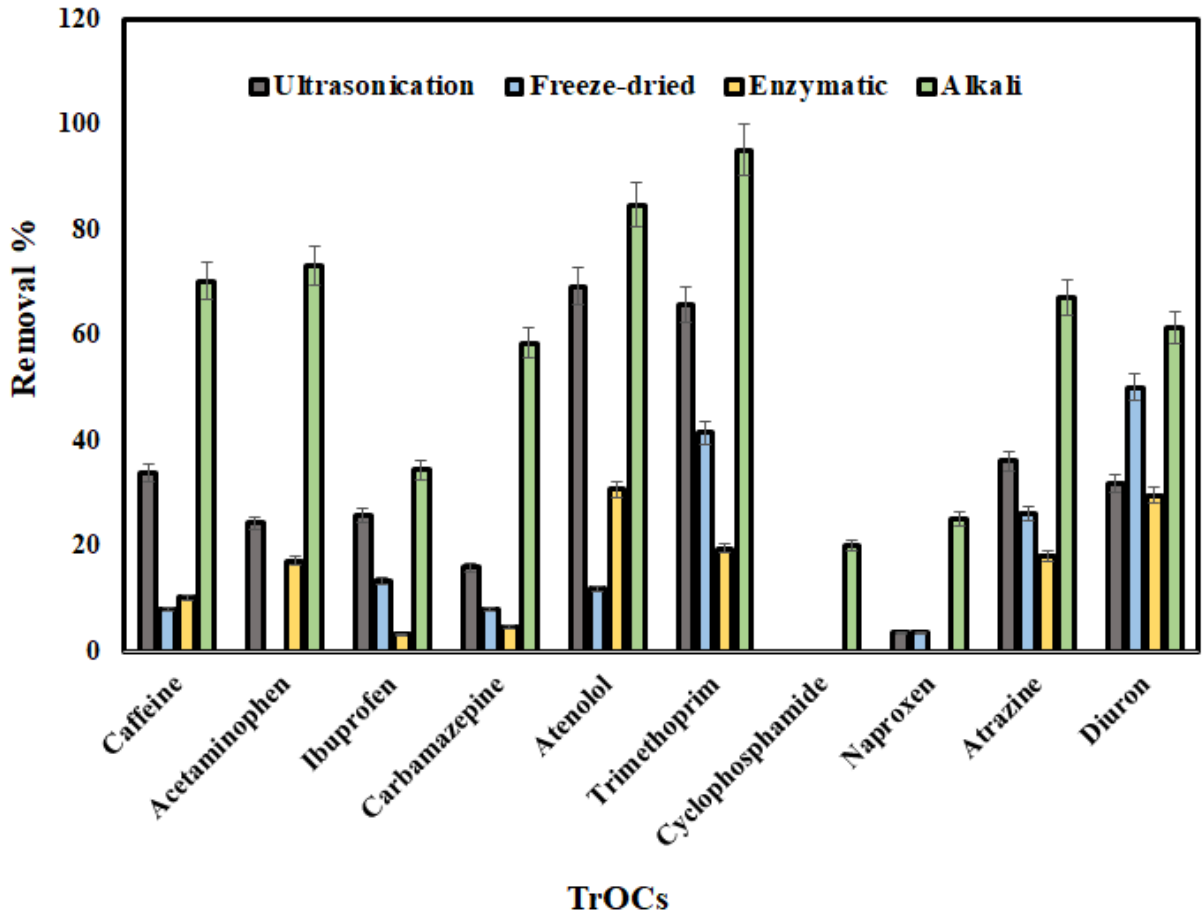

Figure 5.3- TrOCs removal after pretreatments in solid fraction of biosolids slurry

Following alkali PT, sonicated BS slurry reduced the total concentration to 121.4±33.4 µg/kg in the solid fraction, and the removal rate for atenolol and trimethoprim accounted for more than 60% (Figure 5.3). In aqueous fraction, the concentration of carbamazepine and atrazine increased to 7.4 ± 0.3 µg/L and 7.05 ± 0.85 µg/L, respectively, from their initial concentrations (see Annexe 2: Figure 8). Desorption of contaminants from solid fraction is possible due to the destruction of biosolids flocs by hydroxy radicals, which in turn reflects an increase in the aqueous concentration during ultrasonication PT [26]. Freeze-dried and enzymatic PT has lesser impact in removing pharmaceuticals and pesticides in solid fraction while significant variations, which was observed earlier in alkali and ultrasonicated BS slurry of aqueous fraction, was not noticed in enzymatic and freeze-dried pretreated BS slurry. Overall, the order of removal of TrOCs in solid fraction of BS are alkali > ultrasonication > freeze-dried > enzymatic PT.

5.3.2 Bioaugmentation of pretreated biosolids by B.subtilis

5.3.2.1 Reduction in COD and solids content influenced by enzymatic activities

Total solid reduction can be improved by aerobic biodegradation after PT [92]. In this study, TSS was considered as the determining factor of BS volume reduction. Without any PT, 12% TSS was removed after aerobic digestion of BS. As a result of bio augmenting *B. subtilis* to ultrasonicated pre-treated BS slurry, TSS content reduced by 13.5%, which was only 5.2% before bioaugmentation (Figure 5.4(a)). Disaggregation of flocs after ultrasonication PT increased the availability of the components, resulting in enhanced solubilization during aerobic digestion [91]. Intake of proteins, sugar, fatty acids, etc., by microorganisms reduced the organic content, which was reflected in the removal of solid content. During this phase, total proteins and carbohydrates in ultrasonicated BS slurry bioaugmented with *B. subtilis*, reduced to 57% and 79%, respectively (see Annexe 2: Tableau 3). Indigenous microorganisms present in the BS consume the organic matter and convert it into low molecular weight compounds. Addition of new competitive bacteria plays a key role in producing less toxic compounds, due to either competition or acclimatization with the indigenous bacteria by effectively reducing the contaminants load [246]. Moreover, protease, amylase, lipase, and phosphatase, which break protein, carbohydrates and other organic molecules, were found in abundance in the ultrasonicated pretreated BS slurry bioaugmented with *B. subtilis* (Figure 5.5), which played an important role in the conversion of insoluble organic matter to soluble organics and in the subsequent removal of TSS. Further, TSS removal obtained after ultrasonic PT followed by 28 days of aerobic digestion in this present study was low when compared to the established studies [91]. The COD removal after 28 days of digestion in ultrasonicated pretreated BS slurry bioaugmented with *B. subtilis* was about 63% (Figure 5.4(b)). However, COD removal profile was not linear, because during 21st day of incubation, the COD removal dropped from 64% to 55% and concomitantly, SCOD/COD ratio reduced to 0.53 ± 0.04 to 0.40 ± 0.05 (Figure 5.4(c)). During this phase, slight reduction in TSS removal was also observed; but it has to be assumed that probable decrease in COD concentration was due to the release of some organic components by the dead microbes.

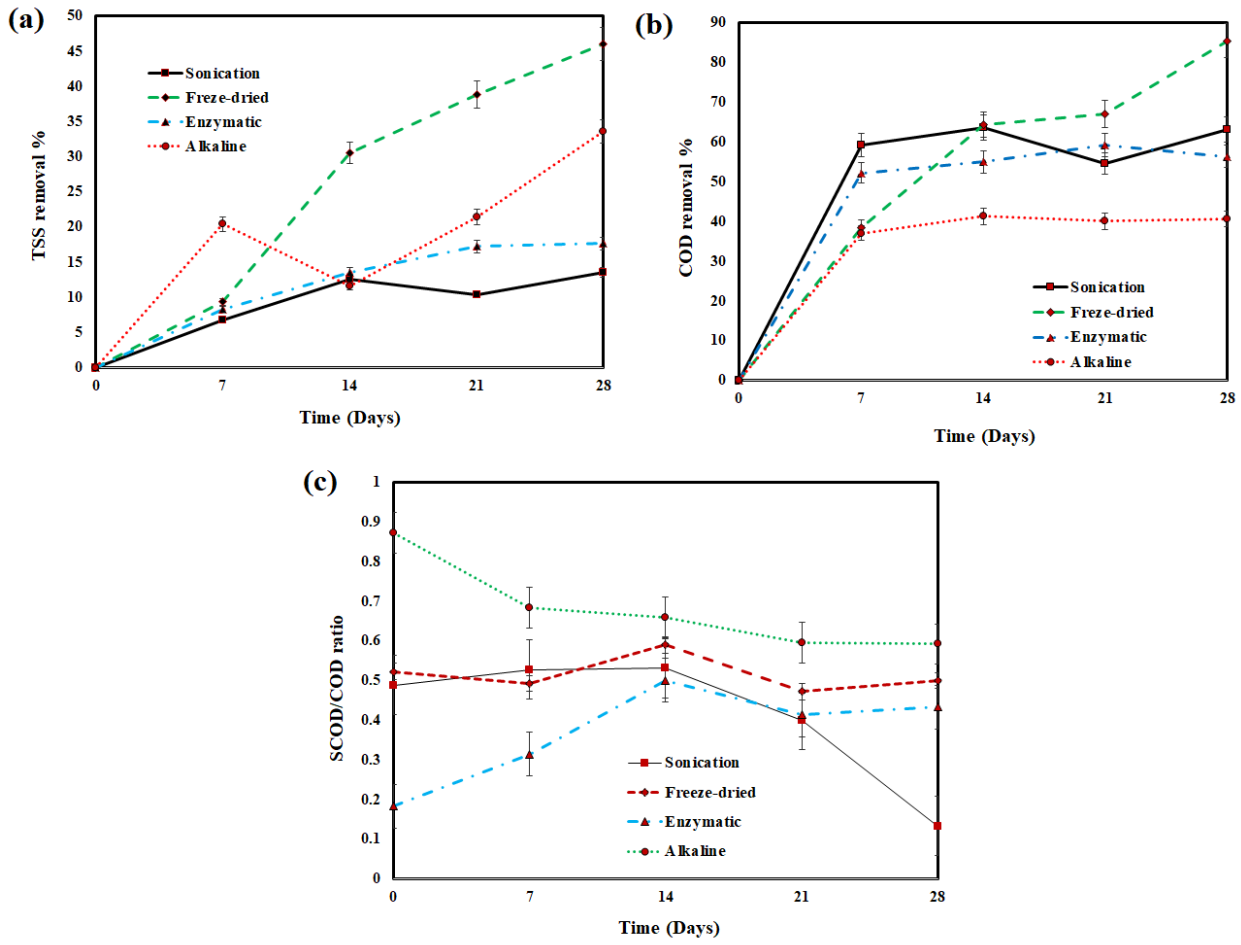

Figure 5.4- (a) TSS removal (b) COD removal and (c) SCOD/COD ratio of after bioaugmentation of *B. subtilis* with pre-treated biosolids after 28 days

The TSS and COD removal in the freeze-dried BS slurry bioaugmented with *B. subtilis* after 28 days of digestion, was about 46% and 85%, respectively (Figure 5.4(a) & 5.4(b)). This reduction in the removal was correlated to the production of extracellular enzymes, which also reduced the total proteins and total carbohydrates to 87% and 85%, respectively (see Annexe 2: Tableau 3). The freeze dried bacterial cells might be reactivated upon effluent addition and the further bioaugmentation with *B. subtilis* bolstered the system to metabolize organics, thus improving the rate of COD and TSS removal [247].

After 28 days of digestion, TSS removal of enzymatic pretreated BS slurry bioaugmented with *B. subtilis* was around 17.6% which was very negligible (0.1%) before bioaugmentation (Figure

5.4(a)). Up to 14 days of aerobic digestion, the TSS removal increased from 0.1% to 13.6%, whereas the SCOD/COD ratio increased from 0.18 ± 0.07 to 0.51 ± 0.04 (Figure 5.4(c)). During this period, the enzymatic activities of protease, amylase, lipase and phosphatase were 7117 ± 105 U/L, 920 ± 41 U/L, 369 ± 23 U/L and 8328 ± 159 U/L, respectively (Figure 5.5). The major cause for this increase in the TSS removal and SCOD/COD ratio is the breakdown of the organic matter by the added enzyme cocktail, along with the enzymes produced by *B. subtilis,* which include protease and lipase, that reduced the solid matter. After 21 days, TSS removal was saturated, which could be due to the complete utilization of the added enzyme cocktail coupled with the reduction in the production of extracellular enzymes by the microbes due to depleted carbon source. It was observed that after 28 days of digestion, the TSS removal was similar to the previous finding of 32% removal after 96 h of digestion with initial sludge concentration of 3.1% [213].

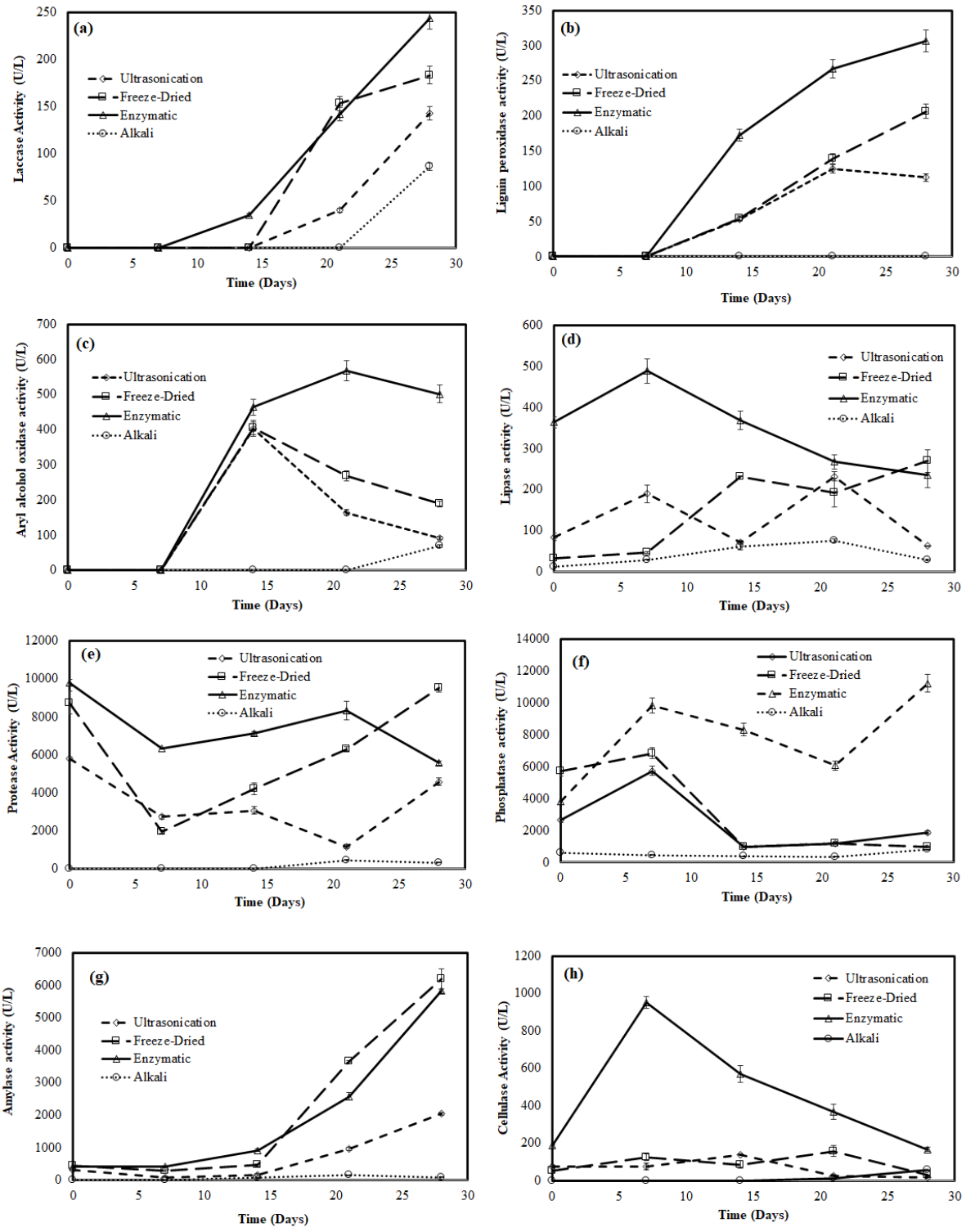

Figure 5.5- Extracellular enzyme profile during bioaugmentation of *B. subtilis* with pre-treated biosolids. ((a) laccase, (b) lignin peroxidase, (c) aryl alcohol oxidase, (d) lipase (e) protease (f) phosphatase (g) amylase and (h) cellulase).

Alkali pretreated BS slurry bioaugmented with *B. subtilis* removed 33.6% and 40.6% of TSS and COD, respectively after 28 days of digestion (Figure 5.4(a) & 5.4(b)). However, the SCOD/COD ratio was reduced to 0.59±0.01 from 0.87±0.11 (Figure 5.4(c)). Moreover, extracellular enzyme activities obtained after 28 days of digestion were 334 ± 22 U/L, 83 ± 8 U/L, 27 ± 2 U/L and 789 ± 29 U/L for protease, amylase, lipase and phosphatase respectively, which were very low when compared to that of all the other pretreated BS slurry bioaugmented with *B. subtilis* (Figure 5.5). Sludge, which consists of a diversity of microorganisms, when introduced to high pH creates an havoc to microbial cells, leading to the production of surface active components [25], high molecular weight components etc., which has possibly inhibited the production of extracellular enzymes by *B. subtilis.* This inhibition might have reduced the breakdown of solid matter into soluble organic, thus decreasing the SCOD/COD ratio. However, the bioaugmented bacteria can uptake the organic matter released during the PT, which reflected the efficient removal of TSS after the digestion. On the contrary, bioavailability did not improve with increased solubilization achieved after PT and the organic matter was not properly utilized [230]. Comparative assessment of results on solids removal of the present study with various PT and/or subsequent aerobic digestion was presented in (see Annexe 2: Tableau 4).

5.3.2.2 Enhanced removal of TrOCs after aerobic digestion

The biodegradation of TrOCs is favorable under aerobic digestion where microbial degradation is the key factor in the removal of TrOCs during such bioprocess [26]. Desorption of TrOCs from sludge flocs was attained by ultrasonic disruption, which increased their bioavailability [26,248], thereby enhancing their removal by the microbes during aerobic digestion. Without any PT, 52% removal of TrOCs was obtained after aerobic digestion of BS by *B. subtilis* for 28 days. The total TrOCs concentration of 10 detected (pesticides and pharmaceuticals) components in solid fraction of ultrasonicated BS slurry before bioaugmentation with *B. subtilis* was 121.4±33.4 µg/kg. After 28 days of digestion, the total TrOCs concentration in the ultrasonicated BS slurry bioaugmented samples was 67.5± 18.45 µg/kg (see Annexe 2:Figure 9). Bolstering of microbial activity during ultrasonication PT with aerobic digestion for 28 days after bioaugmentation with *B. subtilis* improved the TrOCs removal efficiency [26]. In the aqueous fraction after bioaugmentation with *B. subtilis*, caffeine, acetaminophen and trimethoprim were not detected,

but removal of atrazine and carbamazepine was merely less than 5%. Moreover, ibuprofen which was not detected in aqueous fraction of ultrasonicated pretreated BS slurry before bioaugmentation increased to 4.6± 1.6 µg/L (see Annexe 2: Figure 10). TrOCs concentration, which increased in aqueous fraction of ultrasonicated pretreated BS slurry after bioaugmentation with *B. subtilis,* is categorized by negative charged components at pH 7.0. Due to electrostatic repulsion, negatively charged contaminants tend to desorb from solid fraction and transfer to aqueous fraction [26]. There was no ligninolytic activity observed up to 14 days, but at the end of 28 days, laccase, lignin peroxidase and aryl alcohol oxidase activities were 142 ± 14 U/L, 112 ± 9 U/L and 91 ± 9 U/L, respectively (Figure 5.5). Ligninolytic enzymes have been instrumental in the removing pharmaceuticals and pesticides due to their high oxidation potential [198,223]. The total protein and total carbohydrates concentrations were 0.24 ± 0.09 mg/ml and 0.28 ± 0.13 mg/ml, respectively, which were detected even after 28 days of digestion (see Annexe 2: Tableau 3). Microbes uptake contaminants for their survival in dormant state but energy obtained during biodegradation is not sufficient to support microbial growth, when the available nutrient source is only contaminants. Hence, the presence of other carbon sources can lead to the formation of metabolites for the mineralization of components [26]. Out of the detected components, carbamazepine and cyclophosphamide remained unchanged even after the end of the treatment, which is coinciding with studies on no removal during solar irradiation pretreated aerobic/anaerobic digestion on biosolids and less than 25% removal of carbamazepine in aerobic/ irrespective of any treatments while comparing three different WWTPs and as reported due to its persistent nature were reported by [39 ,40]. Thus, the TrOCs removal from solid fraction was observed to be in the order of naproxen > atrazine > diuron > ibuprofen > trimethoprim > atenolol > caffeine > acetaminophen (Figure 5.6).

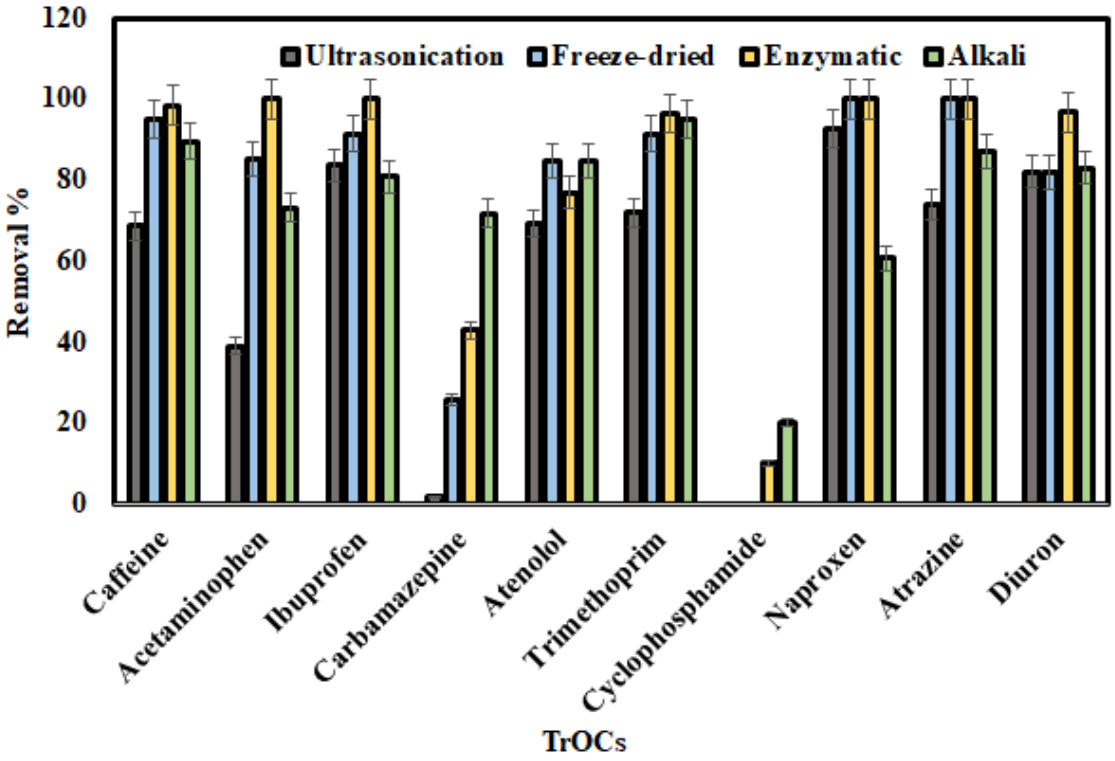

Figure 5.6- TrOCs removal after bioaugmentation of *B. subtilis* with pre-treated biosolids

Freeze-dried BS slurry bioaugmented with *B. subtilis* in solid fraction improved the total TrOCs removal to 83.2% after 28 days of digestion which was 12.8% before bioaugmentation, where 100% removal was incurred for naproxen and atrazine (Figure 5.6). The ligninolytic enzyme activities observed were 183 ± 11 U/L, 206 ± 16 U/L and 184 ± 56 U/L for laccase, lignin peroxidase and aryl alcohol oxidase respectively, even after 28 days of digestion, which might be responsible for the enhanced removal of TrOCs (Figure 5.5). Carbamazepine incurred a removal of around 25%, whereas cyclophosphamide remained unchanged. The removal of the remaining TrOCs was observed to be more than 80% (Figure 5.6). In aqueous fraction after 28 days of digestion, the concentration of TrOCs, such as caffeine, carbamazepine, acetaminophen, trimethoprim, naproxen and atrazine, increased (see Annexe 2: Figure 10). The pH of the freeze-dried pretreated BS slurry bioaugmented with *B. subtilis* changed drastically from 6.21±0.07 to 7.93±0.14 after 28 days of digestion. Transfer of TrOCs from solid fraction to aqueous fraction or vice-versa are possibly due to the variation of pH, which influence the solubility of component in water.

Enzymatic pretreated BS slurry bioaugmented with *B. subtilis* pronounced a total TrOCs removal of 90.1%, which was 80% higher when compared to the solid fraction before bioaugmentation. Out of 10 detected components, 4 components (acetaminophen, naproxen, ibuprofen and atrazine) were completely removed and 3 components (caffeine, trimethoprim and diuron) incurred a removal of more than 96% (Figure 5.6). Cyclophosphamide, which was unchanged throughout all the experiments, accounted to 10% removal while the removal of carbamazepine was about 43%. Presence of atrazine, acetaminophen, trimethoprim, atenolol, caffeine, ibuprofen and carbamazepine were detected in aqueous fraction of enzymatic pretreated BS slurry bioaugmented with *B. subtilis* (see Annexe 2: Figure 10). After the end of 28 days, the maximum ligninolytic enzyme activities observed were 243 ±30 U/L, 306 ± 29 U/L and 500 ± 81 U/L for laccase, lignin peroxidase and aryl alcohol oxidase, respectively (Figure 5.5). While comparing the physical/chemical PT with enzymatic PT, the latter was highly effective in breaking the organic matter into its soluble part, but the bioavailability of the organic matter could not be completely utilized, possibly due to the production of some inhibitory compounds [250,251]. However, the enzymatic PT breaks the organic matter progressively and increase their bioavailability without the additional of inhibitory components, which could establish favorable conditions for the microbes. Thus, even after the complete inactivation of the added enzymes, the removal of contaminants could be achieved by, among others, the oxidative enzymes produced by the microbes with the presence of the available soluble carbon sources.

Alkali pretreated BS slurry bioaugmented with *B. subtilis* had noticeable total TrOCs removal of 83% after 28 days of digestion (Figure 5.6). The contaminants removal for all other pretreated samples increased by 30-80% after bioaugmentation, which was possibly due to the effect of *B. subtilis* and other indigenous microorganisms. However, for the alkali PT, the removal rate increased from 63% to 83% after bioaugmentation with *B. subtilis,* which increased only by 20%. After 28 days, ligninolytic enzyme activities observed were 86 ± 5 U/L and 69 ± 7 U/L for laccase and aryl alcohol oxidase, respectively (Figure 5.5). However, no lignin peroxidase activity was observed throughout the experiment. The production of possible inhibitory components when sludge microbial cell is exposed to high pH might create unfavorable conditions for the bioaugmented as well as the indigenous bacteria, which could possibly lead to the inhibition of extracellular enzymes production [230]. Alkali pretreated BS slurry

bioaugmented with *B. subtilis* has incurred 71% removal of carbamazepine, a recalcitrant compound, which is higher than the removal achieved by all other pre-treated BS slurry after bioaugmentation (Figure 5.6). Removal efficiency of more than 80% for five TrOCs (atrazine, diuron, atenolol, ibuprofen, caffeine) and more than 90% for trimethoprim was obtained. Efficient removal from solid fraction was probably due to production of surface active components during alkali PT which increased the solubility of the selected TrOCs [252], thus, enhancing the bioavailability for microbial actions. Moreover, the obtained removal for carbamazepine and ibuprofen were relatively higher when compared with established studies done by [253], but meanwhile, the removal of naproxen was low. Desorption of PhACs from sludge matrix followed by microbial activity will influence the biodegradation of targeted components, so the balance between the two above mentioned processes will induce a cumulative effect on the biodegradation [254]. In the case of naproxen, bacteria target the methoxy end of the compound; however, the presence of ether bond in methoxy end increases the stability of the compound, which explains the comparatively lower removal of naproxen than carbamazepine and ibuprofen [255]. Atrazine (7.35±0.05 µg/L), caffeine (2.7±0.2 µg/L) and acetaminophen (5.0±2.9 µg/L), which increased in aqueous fraction during alkali PT reduced to (3.95±0.35 µg/L), (0.00±0.00 µg/L) and (3.2±1.1 µg/L) respectively after 28 days of digestion in aqueous fraction of alkali pretreated BS slurry bioaugmented with *B. subtilis* (see Annexe 2:Figure 10). TrOCs which were partitioned to aqueous fraction during alkali PT were readily available to biodegradation for further digestion [26]. Comparative assessment of results on TrOCs removal of the present study with various PT and/or subsequent aerobic digestion was presented in see (see Annexe 2: Tableau 4).

5.4 CONCLUSION

In this present study, we have analyzed the effect of various PT on aerobic digestion of unsterilized BS. Among 72 TrOCs analyzed in the BS, eight pharmaceuticals (157.2±43.4 µg/kg) and two pesticides (18.8±5.8 µg/kg) were detected. The aerobic digestion of BS with *B. subtilis* showed promising removal of TrOCs and TSS. Subjecting the BS to PT processes prior to aerobic digestion was found to be effective for TrOCs and TSS removal. Among the four pre-treatment strategies employed, alkali PT was most reliable before bioaugmentation due to the

maximum removal of TrOCs and a considerable reduction in TSS. Further, among the aerobic digestion of various pre-treated BS, the enzymatic pre-treatment incurred a TrOCs removal of 90%, with 18% TSS reduction. Overall, integrating the pre-treated BS with *B. subtilis* augmented aerobic digestion showed 62-90 % of TrOCs and 13.5-46 % of TSS removal. The key cause could be the production of extracellular hydrolytic and oxidoreductase enzymes during the bioaugmentation process, and their subsequent impact on the reduction of TSS and TrOCs. The treated BS and enzymatic cocktail obtained after the aerobic digestion, further, constitute environment friendly products which can be utilized as land fertilizer and for wastewater treatment.

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CHAPITRE 6

ENZYMES D'INTÉRÊT PRODUITES À PARTIR DE BIOSOLIDES AUGMENTÉS DE CchampignONS ET LEUR UTILISATION POUR LE TRAITEMENT DES PROBLÈMES ENVIRONNEMENTAUX

Enzymes of interest produced from biosolids augmented with fungi and their utilization for treatment of environmental concern

Avant-propos

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Résumé en français : Les travaux actuels se concentrent sur la production de glucose oxydase (GOD) dans des biosolides stérilisés (BS) et des effluents d'eaux usées municipales. Divers paramètres ont été optimisés pour maximiser la production de la GOD et les effets de la biostimulation sur la production de la GOD ont été étudiés en ajoutant au milieu de culture des substances synthétiques. Les études sur les caractéristiques de l'inoculum âgé de 72 h et une concentration de 20% (m / v) a permis de produire environ 6012 U / L de GOD dans des milieux de BS à 25% (m / v). De plus, afin de maximiser la récupération de l'enzyme, l'effet du temps de sonication a été évalué pour la libération de la GOD liée au BS. En utilisant 1000 U / L de GOD produite par *A. niger* par fermention des BS pour l'oxydation d'une solution de glucose à 0,55 M, les résultats ont démontrés une production maximale de H_2O_2 de 216 ppm. Le H_2O_2 produit a été utilisé dans un processus d'oxydation avancé basé sur bio-Fenton pour la dégradation de 15 composés pharmaceutiques actifs.

Mots-clés : Glucose oxydase, Biosolides, Valorisation, Composés pharmaceutiques actifs, Bio-fenton

Abstract : The current work focuses on the production of glucose oxidase (GOD) in sterilized biosolid (BS) slurries containing BS and municipal wastewater effluent. Various parameters were optimized for maximizing the GOD production and the effects of biostimulation on GOD production was investigated by adding synthetic media components. The studies on inoculum characteristics at an inoculum age of 72 h and inoculum size of 20% (w/v) produced high GOD activities of around 6012 U/L in 25% (dw/v) BS media. Further, the effect of ultrasonication time was determined to release BS-bound GOD in order to maximize enzymes recovery. Using 1000 U/L of the BS-based GOD for 0.55 M glucose oxidation produced the maximum H_2O_2 concentration of 216 ppm. The produced H_2O_2 was utilized for bio-Fenton based advanced oxidation process for the partial removal of 15 pharmaceutically active compounds.

Keywords: Glucose oxidase, biosolids, valorization, pharmaceutically active compounds, bio-Fenton

6.1 INTRODUCTION

Glucose oxidase (GOD) is a globular flavoprotein that oxidizes glucose to gluconolactone, which is further hydrolyzed to form gluconic acid and hydrogen peroxide. This redox reaction occurs in the presence of oxygen which acts as an electron acceptor [256] GOD is utilized in several fields and is of economic importance. Among the numerous applications of GOD, glucose detection sensors are widely used and commercialized. The biocatalytic action of GOD leads to the *in-situ* generation of hydrogen peroxide (H_2O_2), which has been majorly utilized in wastewater treatment to treat dye effluents like Malachite Green [257], acid Blue 113 [257] and indigo carmine [258]. Moreover, as the classical Fenton reaction generates non-selective radicals for mineralization of organic contaminants [259], the bio-Fenton reaction can, thus, substitute the classical Fenton process as it does not require an acidic pH of 2.0 [260]. The GOD-mediated bio-Fenton reaction during organic pollutants removal occurs at a neutral pH and the reaction scheme can be described as:

$$C_6H_{12}O_6 + H_2O + O_2 \xrightarrow{GOD} C_6H_{12}O_7 + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\blacksquare} + HO^-$$

$$HO^{\blacksquare} + \text{Pollutant} \rightarrow \text{Oxidation products}$$

GOD play an inevitable role in several industrial application but their high production cost constitute as a major setback [261]. GOD is usually costly due to the high cost-investment in the production of enzymes [256]. By monitoring the entire system starting from fermentation to purification, a considerable amount could be saved by lowering the cost input in formulating the fermentation media. Of the total cost incurred in enzyme production, 30-40% is contributed by the production medium used [19].

According to reports prepared by Hydro-Quebec (2015) the total biomass in the province of Quebec (Canada) was estimated to be 19.5 million tons (dry basis) per year which include agro-industrial and municipal solid wastes [262]. Industrial and municipal biomass can be used as alternate substrates for enzyme production, which will reduce the total production cost [256,261]. There are reports of utilizing agro-industrial biomass like sugarcane bagasse [263], rice straw [264] among others, as well as wastewater sludge [265] as low-cost substrates for

enzyme production. In this context, various agro-biomass had been utilized as substrates for GOD production, and biomass like waste date, wheat bran, wheat gluten and soybean meal were extensively investigated [261].

Biosolids (BS) or dewatered sewage sludge from wastewater treatment plants are one among the potential biomass that could be utilized for enzyme production [266]. In Canada, for example, the annual generation of such biomass is estimated to be around 660 000 metric tons by the Canadian Council of Ministers of the Environment [2]. BS have been reported as one of the sources of energy and it can also be used as a substrate or growth medium for microbial growth since the composition of BS with the metals, minerals and nutrients they contain, it can promote the growth of microbes [267]. GOD can be retrieved from many sources like algae, fruits, bacteria and fungi, especially *A. niger* [256,261].

This study aims at utilizing municipal wastewater treatment plant BS as a substrate for the production of glucose oxidase using the fungal strain *A. niger*. The influence of several parameters like the concentration and initial pH of BS, incubation temperature, agitation speed and the inoculum characteristics (e.g. age, concentration) were studied to obtain the optimum conditions for maximizing the production of GOD. The extracted enzyme was used for the oxidation of glucose to obtain gluconic acid and H_2O_2. The resulting mixture containing the latter two, generated by biocatalysis, was employed in the bio-Fenton process to remove pharmaceutically active compounds (PhACs) from aqueous solution.

6.2 MATERIALS AND METHODS

6.2.1 Materials and Chemicals

All chemicals utilized in this work are of analytical grade. Hydrogen peroxide (30% w/w solution), p-benzoquinone, gluconic acid (50% w/w solution) and D-glucose were from Sigma Aldrich (Saint-Louis, MO, USA). The Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, USA) was used in this study. Deionized water purified and deionized by a Milli-Q purification system (minimum resistivity 18 MX cm; Millipore, Billerica, MA, USA) was used in substrate preparations. 15 PhACs – ketoprofen, mefenamic acid, ciprofloxacin, trimethoprim,

ofloxacin, acetaminophen, ibuprofen, amoxicillin, atenolol, bezafibrate, fenofibrate, caffeine, carbamazepine, cyclophosphamide and indomethacin – were from Sigma Aldrich (Saint-Louis, MO, USA). Methanol and water of LC grade were used in the preparation of standard PhACs solutions and conducting the PhACs degradation study. All reagents of analytical grade of more than 95% purity were utilized. The strain of *A. niger* NCIM 545 was obtained from National Collection of Industrial Microorganisms (Pune, India).

6.2.2 Biosolids characterization

BS and wastewater effluent (WWE) were obtained from a local municipal wastewater treatment plant (QC, Canada) which is based on the activated sludge process and stored at -18°C for further analysis. Similarly, the effluent collected from the WWTP was stored at 4°C. For the preparation of bioslurry, 50 g of the unsterilized biosolids were mixed with 100 ml of unsterilized and undiluted WWE to prepare 50% (w/v) bioslurry, which was then homogenized using a magnetic stirrer at 250 rpm for 4 h. Preparation and characterization of BS slurry is explained in (see Figure 3.2). Bicinchoninic acid test kit from Thermo Scientific (Rockford, USA) was used to analyze the protein concentration [268].

6.2.3 Glucose oxidase production

6.2.3.1 Inoculum preparation

The BS and WWE used for culturing *A. niger* were initially sterilized separately in an autoclave at 121°C and 15 psig (103.4 kPa) for 15 minutes. Glucose (0.5% w/v), yeast extract (0.75% w/v), malt extract (0.25% w/v) (GYM) media, prepared in sterilized WWE, was inoculated with spores of *A. niger* cultured in GYM agar plates. This inoculum was used throughout this study, except while determining the effect of the type of inoculum used. The other type of inoculum was BS-based, prepared by inoculating 5% *A. niger* spores 100 mL of WWE containing 2% (dw/v) BS (sterilized). The pH of both the seed media were adjusted to 4.5 using 0.1 M HCl and all these cultures were incubated in a rotary shaker with agitation at 150 rpm and temperature of 20ºC.

6.2.3.2 Enzyme production optimization

Preliminary experiments were run to study the effect of sterilization, like screening unsterilized and sterilized BS slurry and WWE, to check whether BS could be used as growth medium for *A. niger.* The dried-sterilized biosolids (DS-BS) (mentioned in section 5.4.1) was then mixed with sterilized WWE for obtaining the required concentration. Except for studying the effect of BS concentration, 25% (dw/v) BS slurry was used throughout the study. It was prepared by mixing 25 g of DS-BS with sterilized WWE to a volume of 100 mL and allowed to homogenize overnight on an orbital shaker at 150 rpm in 250 mL Erlenmeyer flasks. This homogenized slurry was screened by inoculating serial diluted samples (10^{-1} to 10^{-6}) on GYM agar plates to ensure the absence of any contamination and further used throughout the production optimization experiments.

The effect of BS concentration, fermentation time, initial pH of the slurry, inoculum characteristics like inoculum type, age and size, as well as the effect of biostimulation by adding optimized synthetic media components (sucrose, peptone, calcium carbonate and magnesium sulphate), were studied by varying one factor at a time. All the optimization experiments were performed in 250 mL Erlenmeyer flasks in triplicates and with respect to abiotic controls. The production media flasks were incubated in a rotary shaker at 150 rpm and at room temperature (20°C). The culture supernatant after centrifugation was checked for the GOD activity.

To study the impact of BS concentration and incubation time, 10% and 25% (dw/v) BS slurry prepared in WWE without varying the initial pH were inoculated with 5% (w/v) of *A. niger* spores solution of 48 h age cultured in GYM media. The enzyme activity was monitored for a period of 144 h. To study the effect of pH, the initial pH of 25% (dw/v) BS slurry in WWE was varied as 3.0, 4.0, 5.0 using 0.1 M HCl and compared with 25% (dw/v) BS slurry of unaltered pH (6.8). This pH adjusted slurry was inoculated with 5% (w/v) inoculum of age 48 h cultured in GYM media and the enzyme activity and the pH profile for a period of 144 h were obtained.

The optimum temperature and agitation speed were determined by varying the temperature from 20°C to 40°C at a constant agitation of 200 rpm and the agitation speed varying from 100 to 250 rpm at 20°C. These experiments were carried out in production media comprising 25% (dw/v)

sterilized BS in WWE inoculated with 5% (w/v) spores of *A. niger* cultured in GYM media for 48 h.

Since biostimulation can be used to enhance enzyme production, the effect of adding the optimized media components [269] to the 25% (dw/v) BS slurry was also studied. This was done by mixing 25 g of DS-BS to make 100 mL of solution using sterilized WWE with the media components and obtain a final concentration of 25% (dw/v) BS slurry, 7% (w/v) sucrose, 0.1% (w/v) $MgSO_4 \cdot 7H_2O$, 0.97% (w/v) peptone and 3.1% (w/v) $CaCO_3$. Moreover, the effect of adding individual media component was investigated in order to reduce the input cost by minimizing the nutrients added. The initial pH was unaltered, inoculum age, size and type were kept constant by inoculating 5% of 48 h inoculum cultured in GYM media during the biostimulation studies.

To comprehend the effect of the type of inoculum, a BS-based inoculum prepared in 2% (dw/v) BS in WWE was used and compared to that of a GYM-based inoculum. This study was achieved by inoculating 5% (w/v) of *A. niger* spores of 48 h inoculum in 25% (dw/v) BS-based sterilized production media prepared by mixing DS-BS with WWE. The effect of inoculum age and size was studied by varying the inoculum age (24–96 h) with constant inoculum size (5%) and by varying the inoculum size from 5% to 20% (w/v) of inoculum age of 48 h. While optimizing the inoculum age and size, the BS media of concentration (25%, dw/v) of DS-BS in WWE, initial pH (unaltered) and the type of inoculum (GYM-based) were kept constant.

In an attempt to maximize the amount of GOD recovered, triplicates of 48 h cultured flask containing 25% DS- BS production media inoculated with 5% w/v spores of *A.niger* (inoculated in GYM media) was subjected to ultrasonication water bath (VWR Symphony-TM, Alberta, Canada) for a period of 30 min with intervals of 5 min. Samples that underwent sonication for every 5 min were centrifuged at 10,000 x g for 15 min. in a benchtop centrifuge (Thermo Scientific Heraeus Mega fuse 8 small benchtop centrifuge, ON, Canada) and the resultant supernatant was used to recover the enzyme associated with the cell and membrane of *A. niger* , as well as the flocs formed in the BS. All studies were conducted in triplicates and reported as average along with the standard deviation.

6.2.4 Enzyme activity measurement

The GOD activity was measured spectrophotometrically at 290 nm on a 96-well plate using a double beam UV-Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corp., CA). The activity measurement was based on the formation of hydroquinone (ε_{290} = 2.31 mM^{-1} cm^{-1}) during the oxidation of 1 M glucose using 0.1% (w/v) p-benzoquinone as an electron acceptor in 50 mM sodium acetate buffer at pH 5.0 and a constant temperature of 25ºC [270]. The absence of GOD activity in the control samples was verified by this method, further facilitating the use of this assay to determine GOD activity in BS-based production media. One unit of activity (U) is defined as the amount of enzyme required to form 1 µmol of product per unit time

6.2.5 Glucose oxidation

The 10 min ultrasonicated culture supernatant was filtered using a 0.22 µm nylon syringe filter, to avoid microbial consumption of glucose during the glucose oxidation experiment. Glucose oxidation was analyzed by varying the concentration of glucose and initial GOD activity. The glucose concentration, varied as 0.1, 0.55 and 1 M and for each concentration of glucose, GOD activities of 100, 550 and 1000 U/L were studied in 50 mM sodium acetate buffer at pH 5.0 and a constant temperature of 25ºC. Glucose oxidation was monitored over a period of 90 min, and the H_2O_2 concentration, glucose conversion and gluconic acid concentration were monitored.

H_2O_2 concentration was estimated spectrophotometrically at 260 nm by reacting the sample with 16.3 mM sodium hexametaphosphate, 67.8 mM cobalt chloride and saturated sodium bicarbonate in constant temperature of 25ºC [271]. Gluconic acid concentration was quantified by titration with sodium hydroxide of known concentrations using phenolphthalein as indicator. The glucose concentration was measured by DNS method at different time intervals [272] and the glucose conversion was determined by the following equation:

$$\%\ \text{of glucose conversion} = \frac{(\text{Initial glucose concentration} - \text{Glucose concentration at time t})}{\text{Initial glucose concentration}} \times 100$$

6.2.6 Bio-Fenton studies

6.2.6.1 Preparation of pharmaceuticals mixture

The stock solution of each PhACs was prepared at a concentration of 1 mg/mL by dissolving 10 mg of PhACs in 10 mL of methanol. This stock solution was stored up until 3 months in the dark at low temperatures (–20°C). The PhACs mixture was prepared at a concentration of 10 µg/mL by taking 1 mL of each PhACs stock of 1 mg/mL and making up the solution to 100 mL using 50 % (v/v) methanol: water. This PhACs mixture solution was further diluted to the required concentration for pharmaceuticals elimination studies. All the PhACs solutions, including the stock and those for the degradation studies, were used in passivated glassware to minimize the adsorption of PhACs [203].

6.2.6.2 Bio-Fenton oxidation for the removal of PhACs

The bio-Fenton process was evaluated for the elimination of PhACs by varying the following parameters: pH, H_2O_2:$FeSO_4$ mass ratio and initial concentration of PhACs. For the PhACs degradation studies of constant contaminant concentration, deionized water was spiked with 15 PhACs to obtain a mixture of final concentration of 500 µg/L of each PhACs. The pH of water was varied as 3.0 and 7.0 to study the effect of pH while the ratio of [H_2O_2:$FeSO_4$] and PhACs concentration was kept at a constant of 50:1 and 500 µg/L, respectively. To study the effect of peroxide: ferrous sulphate ratio, this parameter was set at 2 levels of 1 and 50 while maintaining a constant PhACs concentration of 500 µg/L and a pH of 7. To study the effectiveness of the bio-Fenton process for different concentrations of PhACs, the PhACs concentration was increased from 500 to 950 µg/L. Based on previous studies with other organic contaminants, an attempt to increase the removal efficiency of the bio-Fenton process was performed by adding an additional 50 ppm of H2O2 to the samples with 950 µg/L PhACs after 15 minutes of reaction (doping the samples at 15 min).

For the bio-Fenton study, 0.55 M glucose was oxidized with 1000 U/L GOD and the H_2O_2 produced by the biocatalytic action of GOD on glucose, along with the gluconic acid formed, was used for the bio-Fenton process. After 30 min of reaction, the samples were extracted using Solid Phase Extraction (SPE) which stopped the reaction according to the methodology of [203].

The extracted samples were analyzed by UPLC-MS/MS for determining the PhACs concentrations. All the experiments were done in duplicates and with respect to controls not containing H_2O_2 and $FeSO_4$.

6.2.6.3 Quantification of PhACs

PhACs were extracted and analyzed according to [203] . The analyses were performed using a positive electrospray ionization (ESI+) source in Multi-Reaction-Monitoring mode on an Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, MA) equipped with an Acquity UPLC HSS-T3 column (100 mm × 2.1 mm, 1.8 μm).

6.3 RESULTS AND DISCUSSION

6.3.1. Biosolids and wastewater as a growth substrate

Characterizing the unsterilized 50% bioslurry (50g of unsterilized BS in 100 ml of unsterilized and undiluted WWE) once every 3 months for obtaining the annual composition of the BS, revealed the latter to be a rich source of carbon, nitrogen, phosphorus and hydrogen, along with metal ions of approximately 90 mg/g (see Annexe 3: Tableau.1). The C:N ratio was found to be around 6.6 and the pH of the BS was near neutrality (6.8). The TSS and TDS of the bioslurry were 70.7 mg/mL and 5.5 mg/mL, respectively. Apart from these parameters, enzyme activities like dehydrogenase and esterase were detected in the BS proving the presence of microorganisms in the unsterilized bioslurry (see Annexe 3: Tableau.1) [273].Unsterilized BS, even at 10% w/v, was not successful for growing *A. niger* for the production of GOD (results not shown). This can be attributed to the competition between the indigenous microbes present in the BS and the inoculated fungal species. Similar results of no growth in unsterilized sludge were observed in the previous works, while using 5% inoculum of *A. niger* even in 1% (w/w) TSS sludge due to competition posed by indigenous microbes [274].

In this study, a 5% (w/v) inoculum was chosen which showed survival and further dominance of the inoculated microbe in the BS. On screening BS slurry and sterilized WWE as a growth medium for *A. niger*, no growth was observed in WWE even after incubation for up to 2 weeks.

On addition of 2 g of DS-BS to sterilized WWE, visible growth of the fungi was observed in the WWE and this was further used as an inoculum to test for the production of GOD. For the optimization of GOD production, DS-BS was used, for which visible growth was observed even in 25% (dw/v) BS slurry (see Annexe 3: Figure 10).

6.3.2 Optimization of GOD production using BS as the growth medium

6.3.2.1 Effect of BS concentration and inoculation time

Due to the nutrient availability in the BS, there was a significant production of GOD in the DS-BS and it was observed that the maximum activity of 607 U/L was obtained for 25% (dw/v) BS slurry after 48h of fermentation, which was nearly three times higher than the activity achieved with 10% (dw/v) BS slurry. The GOD activity increased with a rate of 12.64 U/L/h till 48 h, which, however, decreased drastically to 93 U/L at 144 h of fermentation. This decrease in GOD activity could possibly be due to the hindrance in nutrient availability and the changes in the rheology of the media with the progress in fungal growth [192,275]. Moreover, the co-production of proteolytic enzymes might also interfere in denaturing GOD and thus reducing its activity [276]. Contrarily, for 10% (dw/v) BS slurry the highest activity attained was 374 U/L at 72 h with a rate of 5.2 U/L/h, which was 10% lower when compared to that in 25% (dw/v) BS slurry for the same incubation time.

For the 25% (dw/v) BS slurry, an initial decrease rate of 7.66 U/L/h was observed after 48 h, after which the rate decreased to 1.54 U/L/h till 120 h. This can be correlated to the steep decrease in GOD activity to 85% from its maximum activity after 96 h interval, which was better for 10% (dw/v) BS slurry, where it decreased to only 41% of the maximum activity to reach the lowest after 72 h, with a decreasing activity rate of nearly 1 U/L/h. However, after 120 h, a drastic change in the rate of decrease in GOD activity was observed in both the 25% (dw/v) BS slurry and 10% (dw/v) BS slurry, which was found to be 10.58 U/L/h and 5 U/L/h respectively till 144 h.

While comparing 25% (dw/v) BS slurry with 10% (dw/v) BS slurry, the enzyme activity in 25% (dw/v) BS slurry increased abruptly with incubation time to reach the maximum activity at 48 h,

after which it decreased steeply. On the other hand, for 10% (dw/v) BS slurry, there was a steady and gradual increase in the activity which reached the maximum at 72 h and thereafter, it decreased slightly to reach the lowest peak. The presence of high nutrient level in 25% (dw/v) BS slurry when compared to 10% (dw/v) BS slurry can be a possible reason for maximum activity within short interval in 25% (dw/v) BS slurry when compared to the latter (Figure 6.1).

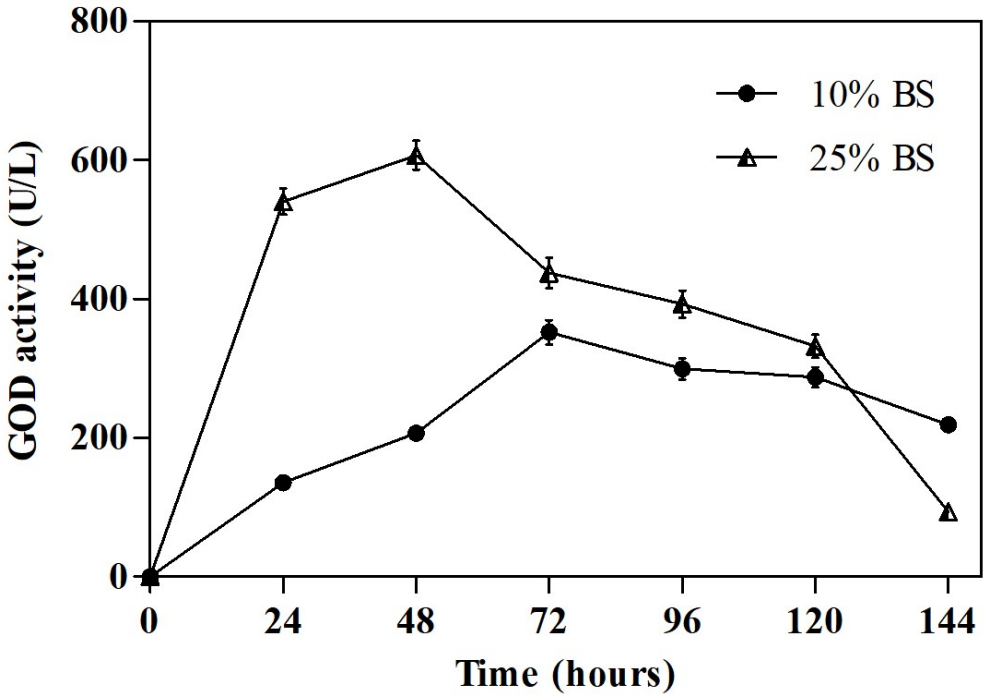

Figure 6.1- The effects of 10% and 25% (dw/v) BS slurry of unaltered pH (6.8), prepared by mixing DS-BS in WWE, on the production of GOD with respect to time using a GYM-based 48 h inoculum of 5%. All studies were conducted in triplicates and reported as average along with the standard deviation

6.3.2.2 Effect of initial pH of BS slurry

The initial pH (pH 6.8) of the 25% (dw/v) BS slurry prepared in WWE was altered to acidic conditions and then the effect of pH was studied by inoculating the sterilized 25% (dw/v) BS media with 5% w/v of 48 h *A. niger* inoculum [277]. The enzyme production decreased with a decrease in the initial pH of the BS media (Figure 6.2(b)). In the synthetic media utilized for the production of GOD, $CaCO_3$ is used to buffer growth media to a pH of 5.8 during fermentation

[278]. The growth of fungi usually leads to a decrease in pH [275]. But acidic pH has an antagonistic effect on the production of GOD [269], which was also evident in our samples. Though the growth of the fungi was observed in samples adjusted to pH 3.0, no GOD activity was noticed beyond 48 h.

In the pH unaltered samples (initial pH 6.8), the pH of the production media was monitored for a period of 72 h (Figure 6.2(b)). The pH of the 25% (dw/v) BS slurry decreased with respect to time and dropped to around 5.6 by the 12^{th} h and stayed around 5.0 until 48 h when the maximum enzyme activity of 1391 U/L was observed (Figure 6.2(a)). The pH dropped significantly after the peak at 48 h after which the GOD production decreased significantly. Fungal growth usually leads to a decrease in the pH of the medium due to accumulation of acidic metabolites [279], which were observed even in fungal treatment of raw wastewater and activated sludge respectively [274].

6.3.2.3 Effect of temperature

Varying the incubating temperature of the BS production media from 20°C to 40°C (Figure 6.2(c)), showed a maximum enzyme activity of 2241 U/L at 30°C, which was 8 % and 149% higher when compared to the highest peaks at 20°C and 40°C, respectively. More than 2000 U/L was obtained at the two lower temperatures, whereas only around 900 U/L was yielded at 40°C. This suggests that temperatures higher than 30°C have a negative impact on GOD activity. With *Penicillium sp.* for GOD production, 25-35°C was found to be the optimum temperature [278]. Fungi mainly grows and thrives well at lower temperatures, but at higher temperatures enzyme production from the fungi could be greatly affected due to metabolic heat and growth inhibitions [275]. Since almost similar results were obtained at 20°C and 30°C, further experiments were performed at 20°C.

6.3.2.4 Effect of mixing

The size and diameter of the fungal biomass is an important parameter for determining the enzyme production of the fungi [275]. While studying the effect of agitation speed on GOD

production, an agitation of 150 rpm yielded the maximum enzyme activity of 2262 U/L at 48 h which was 2.5 times and 5.6 times higher compared to 250 and 100 rpm respectively (Figure 6.2(d)). Agitation speed plays a major role in determining the availability of oxygen, nutrients, inducers to the micro-organisms. Nutrient and oxygen availability, and the morphology of the fungal biomass are determined by the agitation speed and rheology of the medium. The absence of mechanical agitation lowers the oxygen transfer efficiency as well as the mixing performance [280]. Oxygen is essential for the growth of the fungal culture; but increased mycelial growth may have an adverse effect. Thus, 150 rpm could have resulted in better rheology and homogeneity of the medium when compared to 100 rpm and 250 rpm [281]. The agitation speed also directly impact the cells by creating shear stress on the cell membranes and high shear could causes disruption of cells, which probably yielded comparatively lower enzyme production at 250 rpm [160].

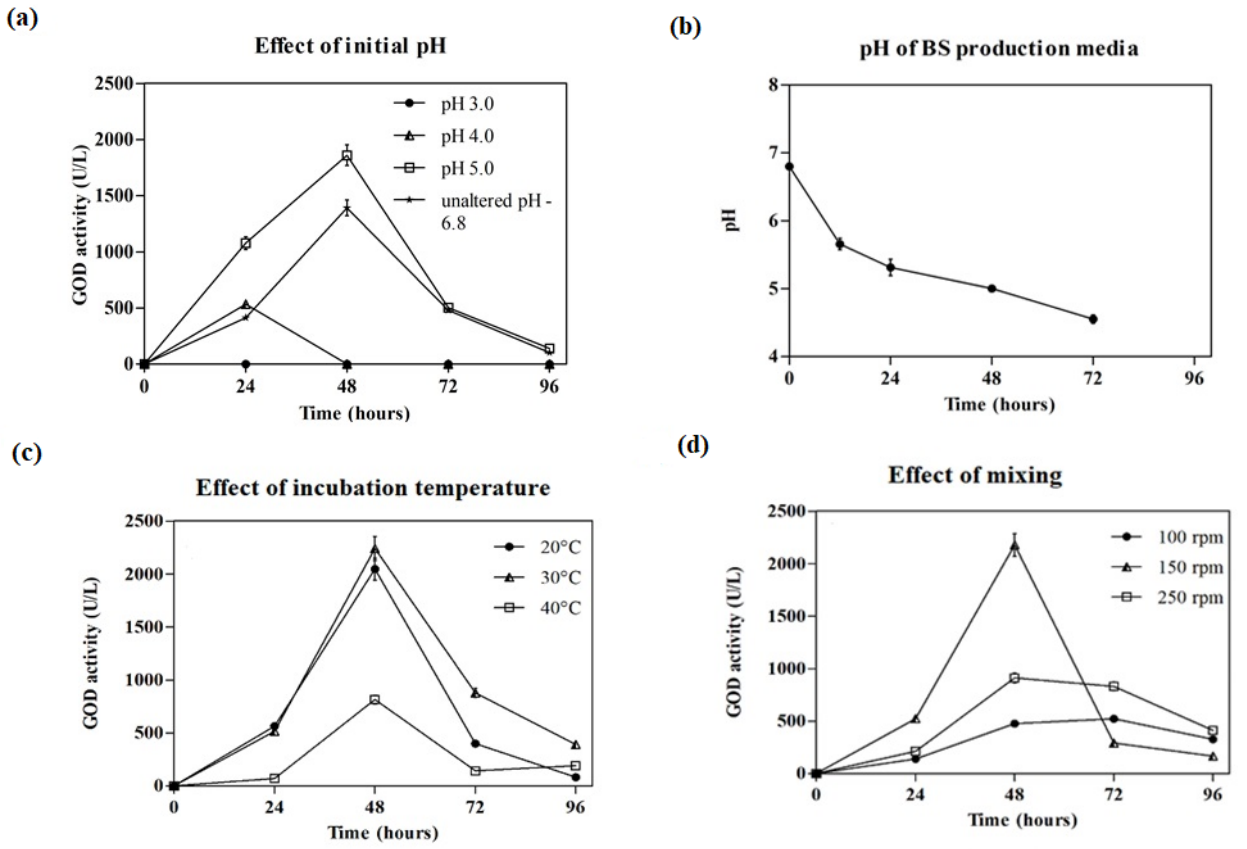

Figure 6.2- (a) Effects of initial pH of BS media on GOD production (b) Monitoring of pH changes during the GOD production. The effect of varying the initial pH of the 25% (dw/v) BS slurry to 3.0, 4.0, 5.0 and unaltered pH 6.8, (c) incubation temperature, (d) mixing on the production of GOD in 25% (dw/v) BS slurry of unaltered pH (6.8), prepared by mixing DS-BS in WWE, on the production of GOD while using a constant GYM-based inoculum of 48 h and 5%. All studies were conducted in triplicates and reported as average along with the standard deviation

6.3.2.5 Effect of biostimulation by adding synthetic media components

With an attempt to increase the enzyme production and obtain maximum enzyme in minimum time, the effect of biostimulation, by adding the optimized synthetic media components was also studied. Using the optimized media (sucrose 7%, calcium carbonate 3.1%, peptone 0.97% and magnesium sulphate 0.1%), the GOD activity in the 25% (dw/v) BS slurry increased two-fold and a maximum enzyme activity of 2327 U/L was obtained in 24 h of fermentation (Figure 6.3(a)). In order to minimize the inclusion of synthetic components in the growth medium, thereby reducing the cost, the effect of individual chemicals added was also studied (Figure

6.3(b)).The supplementation of the BS with calcium carbonate produced results similar to that of the whole media and a maximum activity of 2209 U/L was achieved in 24 h of fermentation. Moreover, its peak was 5.5,4.8 and 3.1 times higher when compared to sucrose, peptone and magnesium sulfate respectively at 24 h. The addition of calcium carbonate not only prevented the lowering of pH, but was also reported as an inducer, causing a metabolic shift from Embden-Meyerhof-Parnas pathway (glycolysis) to GOD production by decreasing the production of 6-phosphofructokinase (6-PFK) in the microorganism. The decrease in 6-PFK production was previously reported in calcium carbonate-added samples which explained the importance of the addition of calcium ions in GOD production [277]. After 24h of fermentation, the enzyme activity decreased linearly to record the minimum activity of 614 U/L at 96h. Impact of the following nutrient sources, sucrose, magnesium sulfate and peptone, supplemented individually, resulted in maximum activities of 1879, 835 and 1629 U/L, respectively, after a 48h cultivation period, after which the activity decreased drastically and no apparent activity was observed at 96h, except with magnesium sulfate, which did not endure any enzyme activity at 72 h. In comparison to the addition of whole media, the effect of adding these compounds was not much pronounceable. The amendment of carbon and nitrogen source into the 25% (dw/v) BS slurry led to an increased enzyme activity by 48 h when compared to the unamended BS. The addition of sucrose produced a maximum of 1879 U/L of GOD activity which can be explained by the activation of the fungi to produce GOD in the presence of high glucose concentration – either directly or through sucrose amendment [269,279].

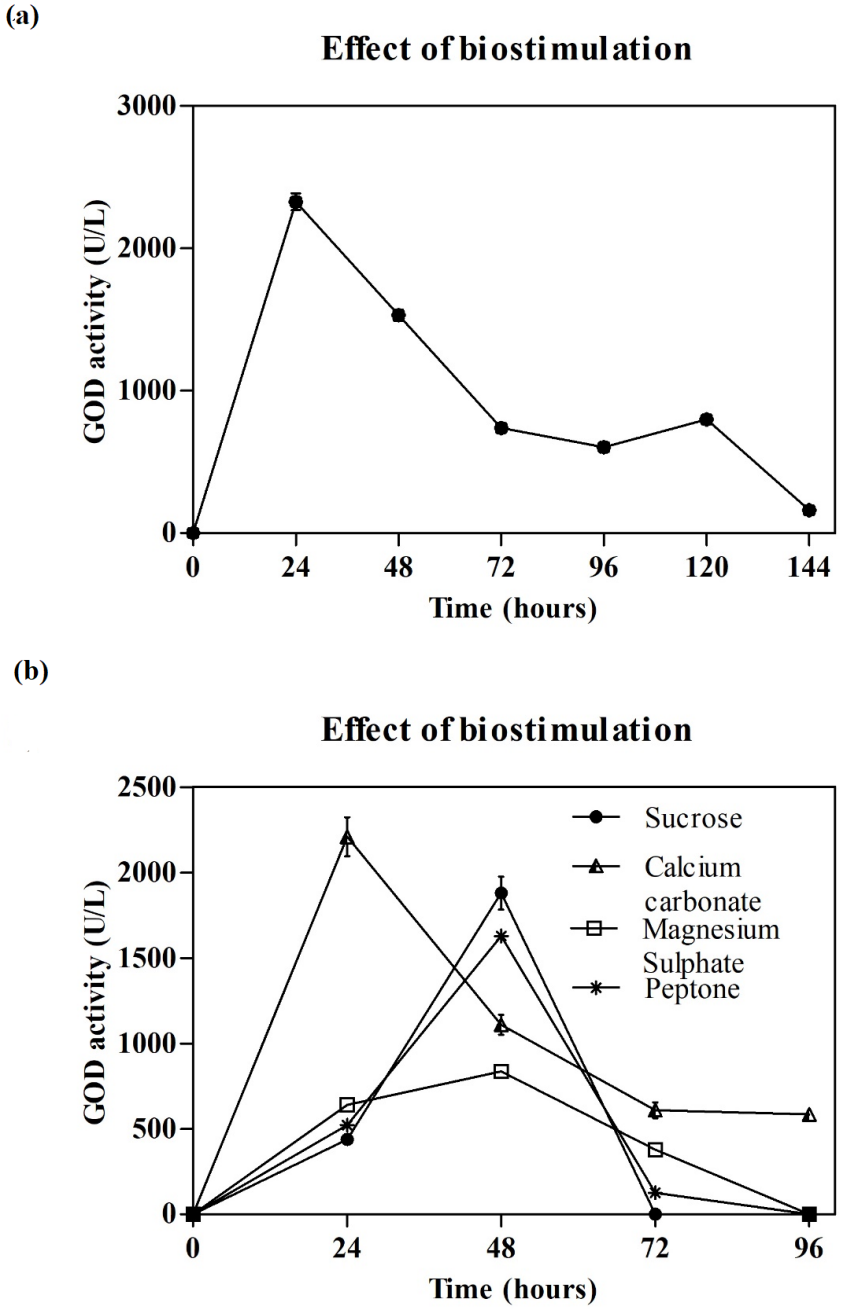

Figure 6.3- Effect of biostimulation on GOD production by (a) adding the optimized media components (b) adding the media components individually. Effect of adding media components – sucrose (7%), peptone (0.97%), magnesium sulphate (0.1%) and calcium carbonate (3.05%) together and individually to the 25% (dw/v) BS slurry of unaltered pH (6.8), prepared by mixing DS-BS in WWE, on the production of GOD while using a constant GYM-based inoculum of 48 h and 5%. All studies were conducted in triplicates and reported as average along with the standard deviation

6.3.2.6 Effect of the type of inoculum

In an attempt to minimize the cost of inoculum preparation, *A. niger* spores cultivated in 2% (dw/v) BS were also tested for GOD production in 25% (dw/v) BS slurry. The use of similar media components for both the inoculum and production media improves the acclimatization of the strain to components present in BS which is subject to variation and reduces the osmotic stress faced by the microorganisms after inoculating at higher concentrations of BS. Increased growth rate of *Bacillus* with reduced lag phase was noted in sludge while using a sludge-based inoculum [192]. Our study dictates that GOD produced with GYM inoculum was 1.4 times higher than that from 25% (dw/v) BS slurry, where a maximum of 858 U/L was obtained in 48 h (Figure 6.4(a)).

6.3.2.7 Effect of inoculum age and size

Using 5% inoculum of varying age in GYM medium, for 24 to 96 h, a maximum activity of more than 1000 U/L was attained in 48 h. (Figure 6.4(b)) A comparatively higher activity of 1618 U/L was obtained while utilizing an inoculum of age 72 h. On varying the inoculum size from 5% to 20%, a maximum activity of 6012 U/L was detected in 48 h with an inoculum size of 20%, which is nearly six times higher than that observed with 5% inoculum (994 U/L) (Figure 6.4(c)). This could be due to the better utilization of substrate at higher inoculum sizes [282]. After 96 h of fermentation , no GOD activity was detected which can be due to high fungal growth leading to lesser nutrient availability [283].

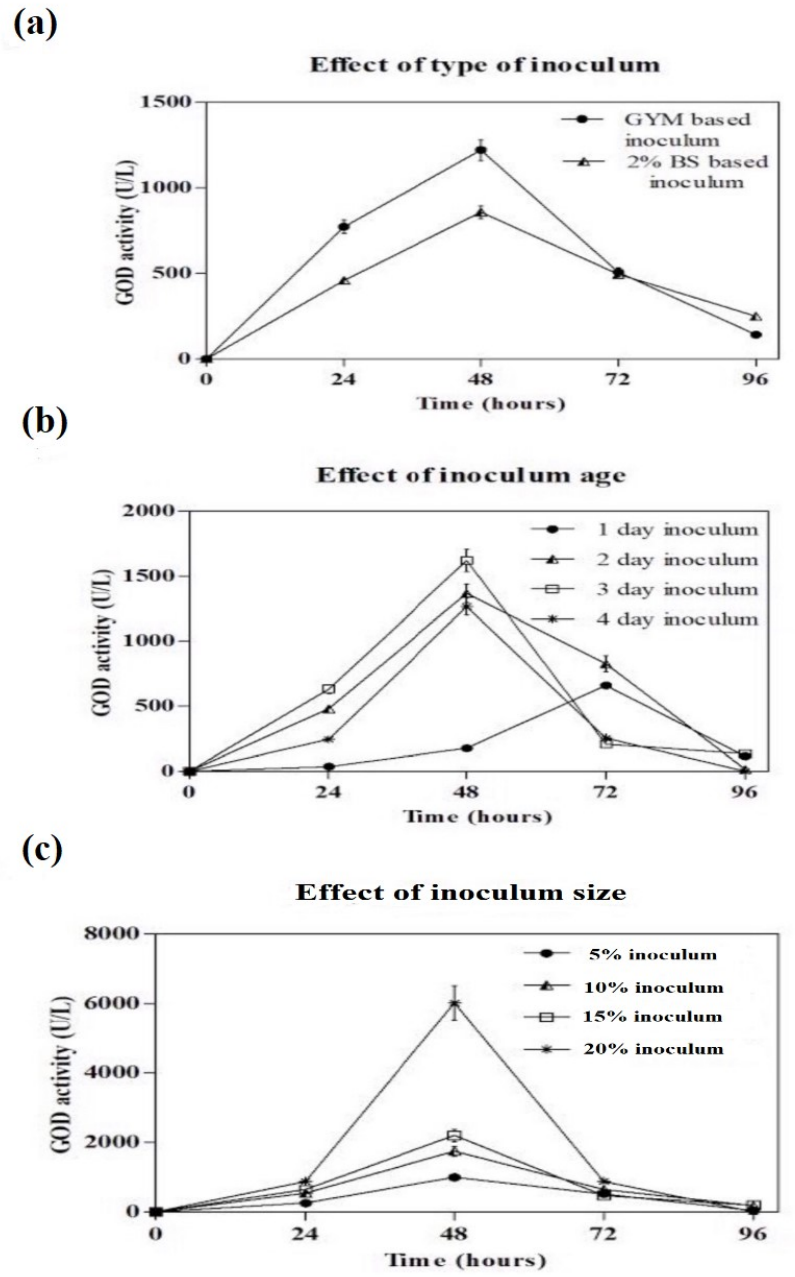

Figure 6.4- Effect of the (a) inoculum type, GM vs BS (b) inoculum age (c) GM inoculum size used on GOD production. The effect of inoculum characteristics while using 25% (dw/v) BS slurry containing DS- BS and WWE on inoculating 5% inoculum of varying age of 1, 2, 3 and 4 days cultured in GYM media and by using a 2-day inoculum prepared in GYM media inoculated of varying sizes as 5%, 10%, 15% and 20%. The effects of inoculum type by inoculating the 25% (dw/v) BS slurry containing DS-BS and WWE with a 2-day inoculum of 5% cultured in GYM-based media and a 2-day inoculum of 5% cultured in 2% (dw/v) BS-slurry containing DS-BS and WWE. All studies were conducted in triplicates and reported as average along with the standard deviation

6.3.3 Effect of ultrasonication to recover enzyme

On sonicating the 25% (dw/v)-BS-based production medium for 5 min in an ultrasonic bath, the enzyme activity significantly increased from 1105 U/L to 1778 U/L. Also, the protein concentration increased from 10.6 to 11.2 mg/ml. Both the enzyme activity and the protein concentration were maintained at higher values with sonication up to 10 min, after which both decreased to levels lower than the initial values (Figure 6.5). Prolonged sonication leads to higher temperature which generates free radicals due to ionization of water, thereby inactivating the enzyme [284] and decreasing the enzyme activity. Around 1.5 times higher GOD activity was recovered by sonicating up to 10 min (specific activity increased from 0.10 to 0.16 U/mg), after which the activity recovered decreased drastically with a rate of 174 U/L/min till 15 min followed by 31.7 U/L/min till 20 min, where no activity was noted. This initial improvement in recovery of the enzyme could be attributed to the location of the enzyme in the fungal strain. In *A. niger*, most of the enzymes are located in the cell wall and cytoplasm rather than in the extra-cellular production media [285]. Apart from the cell wall-bound enzyme, fungi forms agglomeration in BS and increases the formation of flocs, within which the enzyme may get trapped [275]. The cell wall structure of *Aspergillus sp.* is rigid due to the presence of chitin (10-20%), which requires longer sonication time for disintegrating the fungal cell wall [284]. Thus, ultrasonication helps in recovering the membrane and mycelia-bound enzyme.

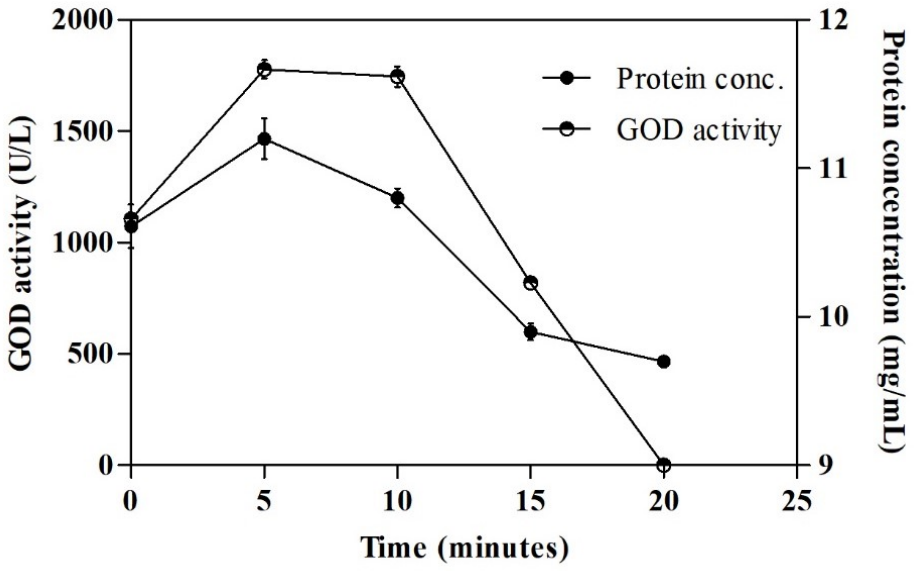

Figure 6.5- Effect of ultrasonication on recovery of GOD. Protein concentration (mg/mL) and GOD activity (U/L) during ultrasonication of 25% (dw/v) BS slurry for GOD production, with respect to time during sonication for 25 minutes. All studies were conducted in triplicates and reported as average along with the standard deviation.

6.3.4 Glucose oxidation using GOD from BS

The effect of glucose concentration (0.1 M, 0.55 M and 1 M) and GOD activity (100 U/L, 550 U/L and 1000 U/L) on H_2O_2 production was studied (see Annexe 3: Figure 11(a)). In all the samples, a linear rise in the H_2O_2 concentration was observed with increase in time up to 90 min, which later decreased. The maximum H_2O_2 concentration obtained with 0.55M glucose and 1000 U/L GOD was 216 ppm, which was found to be 1.3 times and 2.7 times higher when compared to 0.1M and 1M -1000 U/L GOD activity at same detection time of 90 min. The overall maximum concentration of H_2O_2 of 216 ppm was reached after 90 min of reaction when around 42% of 0.55 M glucose was oxidized with 1000 U/L GOD. With the other concentrations of glucose tested, the maximum H_2O_2 concentration 1844 ppm with 0.1 M glucose undergoing 12.4% conversion and 78 ppm with 28.3% conversion of 1 M glucose. The low concentration of H_2O_2 can be attributed to the destabilization of H_2O_2 in the presence of catalase and peroxidases and interactions with dissolved iron or organic compounds in the growth medium [286]. GOD and catalase are known to exist both as extracellular and in the membrane of the fungi; hence

sonication would further release catalases [287]. The purification of GOD from catalase requires a high cost investment [287]. The existence of catalase and peroxidases in the reaction mixture, thus, greatly affects the yield of H_2O_2 and further, the stability of H_2O_2 in the reaction mixture.

The glucose conversion obtained with 1000 U/L, compared to 100 U/L, was lesser after one hour of reaction (see Annexe 3: Figure 11(b)). For all the tested concentrations of glucose, the glucose conversion increased with time up to 90 min of reaction but never exceeded 50 % conversion. On further reaction time up to 24 h, the glucose conversion stabilized, and no increase was observed; whereas the 113 ppm of H_2O_2 detected in the samples was much lower. The low conversion of glucose can be a possible effect of enzyme deterioration due to H_2O_2 being detrimental to many proteins, including enzymes like GOD [279]. H_2O_2 can deactivate enzymes and this enzyme deactivation caused by the formed H_2O_2 can be minimized by stabilizing the enzyme by immobilization [288,289].

6.3.5 Bio-Fenton oxidation to remove pharmaceuticals

6.3.5.1 Effect of pH

After 30 min of bio-Fenton reaction for a H_2O_2:$FeSO_4$ ratio of 50:1, all the compounds except for atenolol and ciprofloxacin were removed partially at both the tested pH of 3.0 and 7.0 (Figure 6.6). The maximum removal was observed for amoxicillin which was removed by about 44%. The target contaminants were only partially removed, and the removal efficiencies were below 50 %. The better removal of compounds at neutral pH by the bio-Fenton process can be explained based on the availability of (Fe^{2+}). Even if performed at pH 7.0, the addition of GOD treated glucose as the H_2O_2 source in the bio-Fenton process provides the platform for chelation of iron to occur. This gluconic acid provided by the oxidation of glucose, aids in iron chelation [290,291]. Thus, bio-Fenton provides an efficient method of utilizing the neutral pH of water to undergo Fenton reaction to remove contaminants.

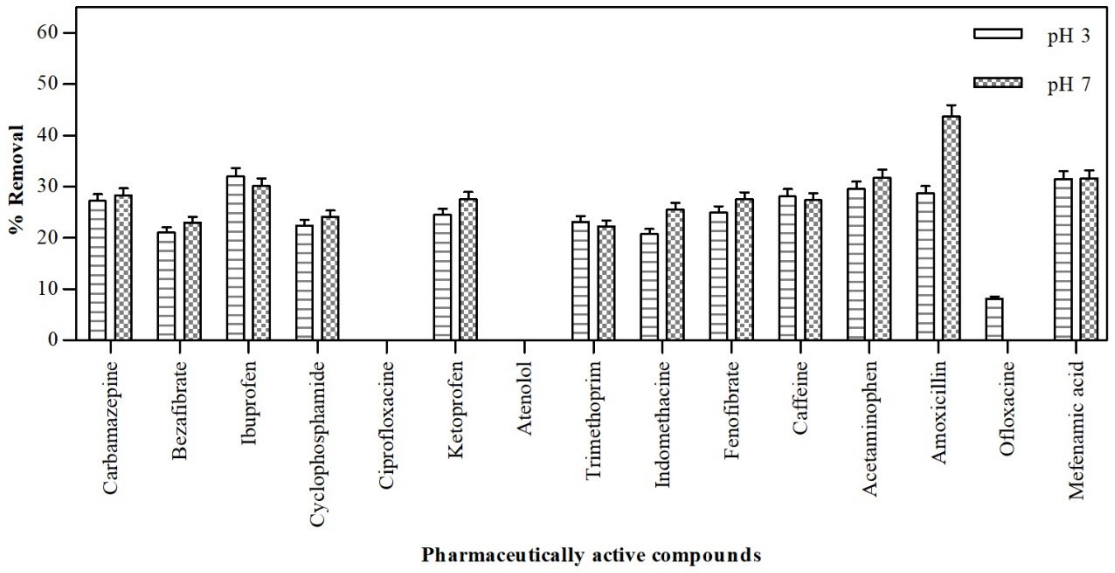

Figure 6.6- Effect of pH on removal of PhACs from aqueous solutions using bio-Fenton oxidation. The removal of the 15 PhACs existing as mixtures in aqueous solutions at a concentration of 500 µg/L (each) and [H_2O_2:$FeSO_4$] of 50:1 at acidic (pH 3.0) and neutral pH (pH 7.0). All studies were conducted in triplicates and reported as average along with the standard deviation.

6.3.5.2 Effect of H_2O_2:$FeSO_4$ ratio

On studying the effect of the peroxide: ferrous sulfate ratio, higher removal was observed while using a H_2O_2: $FeSO_4$ ratio of 50:1 rather than 1:1 (Figure 6.7). Since the amount of [Fe^{2+}] was increased to 20 mg/L while utilizing a ratio of 1:1 and the increased availability of ferrous ions at neutral pH by gluconic acid, the generated hydroxyl radicals could possibly be scavenged by Fe^{2+} ions [292]. Even if the amount of Fe^{2+} added was lower while using a ratio of 50:1, the high amount of H_2O_2 and increased chelation of Fe^{2+} by gluconic acid can be accounted for the better removal of PhACs at neutral pH [293]. It is less feasible to conclude one ratio as the optimum ratio for degradation of PhACs since individual PhACs are unique in structure and properties and the target water contains a mixture of PhACs. Such differences were observed in dye degradation in wastewater where the degradation of dispersions were interfered by the presence of dye auxiliaries [293]. Apart from this, for different contaminants, different optimum ratios were

reported. This denotes that the best H_2O_2:$FeSO_4$ ratio depends on the type of organic component targeted during the (bio)fenton process [293].

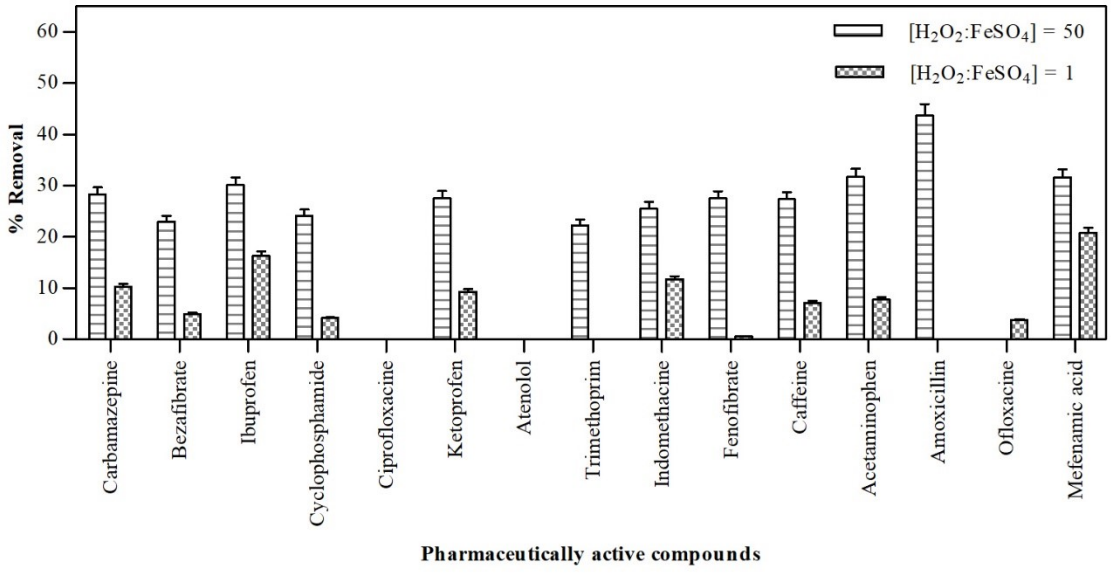

Figure 6.7- Effect of [H_2O_2:$FeSO_4$] on removal of PhACs from aqueous solutions using bio-Fenton oxidation. The removal of the 15 PhACs existing as mixtures in aqueous solutions at a concentration of 500 µg/L (each) and neutral pH (7.0) at varying [H_2O_2:$FeSO_4$] of 50:1 and 1:1. All studies were conducted in triplicates and reported as average along with the standard deviation.

6.3.5.3 Effect on increased concentration of PhACs

Based on the results obtained, the experiment conditions were set as 50:1 for the H2O2:FeSO4 ratio and the pH as 7.0. To test the efficiency of the bio-Fenton process, the concentration of PhACs were almost doubled, from 500 µg/L to 950 µg/L. Removal was observed only for few nonsteroidal anti-inflammatory drugs – ibuprofen, ketoprofen and mefenamic acid, and fenofibrate, but the removal was less than 10 %. H_2O_2 was observed as a limiting factor, in which the removal of dye compounds remained unchanged after reaching a particular concentration [293]. Based on previous results obtained with the removal of TCE (unpublished work), an attempt to increase the removal of PhACs was performed by adding additional 50 ppm of H_2O_2 after 15 minutes of the reaction. This led to the partial removal of 12 out of 15 PhACs at 950 µg/L concentration. The removal efficiency was almost similar to the removal obtained with 500

µg/L PhACs (Figure 6.8). This increase in the removal of the contaminants can be explained by the generation of HO• with the increase in H_2O_2 concentration [294]. Thus, this proves that the concentration of H_2O_2 is an important rate limiting factor that has to be monitored and optimized, and the ratio of H_2O_2:$FeSO_4$ has to be altered for different concentrations of the contaminant(s).

Even if removal is observed, the low removal of compounds by the bio-Fenton process can be explained based on the reaction used for the generation of H_2O_2. Since complete conversion of glucose was not achieved while using GOD, the presence of unreacted glucose may hinder the oxidation of the target contaminants. The presence of unreacted glucose and more than one organic target contaminant in combination with the non-selective nature of the hydroxyl radical could be stated as one of the reasons for the less than 50% removal of PhACs in the study [295].

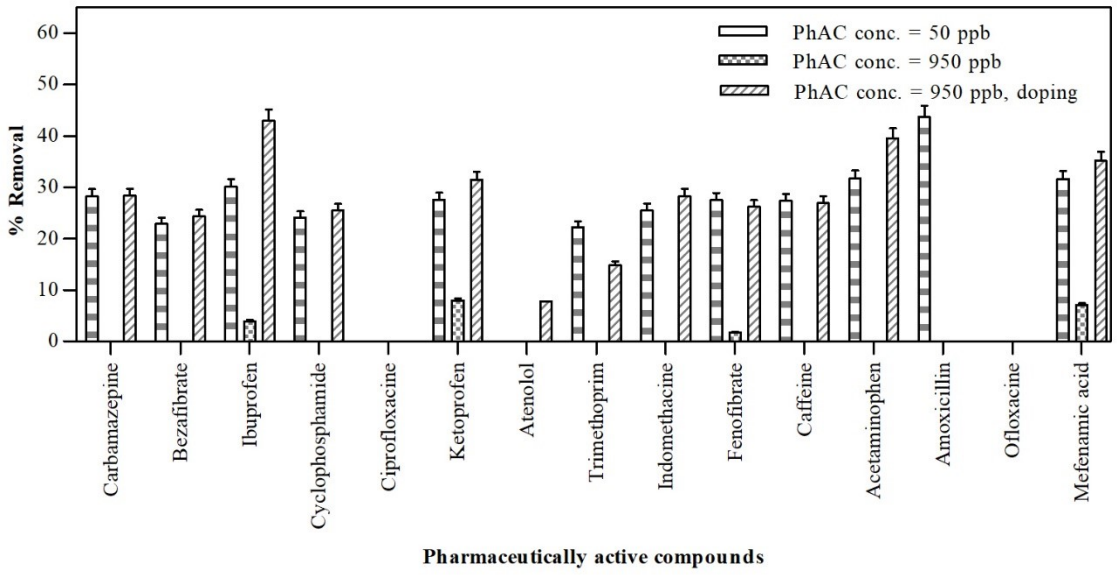

Figure 6.8. Removal efficiency of bio-Fenton oxidation of varying concentrations of PhACs from aqueous solutions and effect of doping H_2O_2 on bio-Fenton oxidation. The removal of the 15 PhACs existing as mixtures in aqueous solutions at a concentration of 500 µg/L (each) and 950 µg/L (each) at neutral pH (7.0) and constant [H_2O_2:$FeSO_4$] of 50:1. Effect of adding 50 ppm of H2O2 after 15 minutes of reaction (doping) on removal of 950 µg/L (each) PhACs. All studies were conducted in triplicates and reported as average along with the standard deviation.

6.4 CONCLUSION

This study reported considerable GOD activities using synthetic media combined with biosolids and effluent from a municipal wastewater treatment plant. It confers valuable information about the impact of inoculum characteristics, providing an alternate, cost-efficient method to improve the GOD production rather than biostimulation. Further, the H_2O_2 generated by the oxidation of glucose by GOD was used as a Fenton reagent in an advanced oxidation – bio-Fenton process, for the removal of organic compounds. However, the constraints caused in this study could be overcome by immobilizing the enzyme to reduce enzyme deactivation, and further perform a complete optimization of the factors influencing the bio-Fenton removal of the target contaminants. The future work could focus on performing the bio-Fenton reaction with reduced glucose, maximizing the conversion of glucose, and optimizing the time required for the reaction. Thus, this study proposes a biocatalysis-based advanced oxidation process as a cost-efficient bioremediation strategy to improve recalcitrant organic compounds removal in treatment plants along with assaying the role of H_2O_2 in bioremediation.

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CHAPITRE 7

CONCLUSION GÉNÉRALE ET PERSPECTIVES

Si le processus de production d'enzymes hydrolytiques et oxydantes par divers microorganismes (bactéries, champignons, etc.) dans différents milieux nutritifs a été étudié par plusieurs auteurs, la combinaison de biosolides municipaux et des eaux usées comme milieu de culture et de la souche bactérienne/fongique produisant le cocktail d'enzymes oxydantes et hydrolytiques n'a pas été largement utilisée et constitue la pierre angulaire de ce projet de recherche. Comme cette thèse vise à développer une approche bio intégrée permettant la réduction et le traitement des biosolides tout en produisant des enzymes pouvant être valorisées pour le traitement de l'eau, afin que l'approche de recherche produise des biocatalyseurs à faible coût d'intérêt industriel et atteigne terme, durabilité. Le présent travail est un procédé respectueux de l'environnement pour convertir les déchets solides en un substrat intermédiaire pour la production de produits à valeur ajoutée. La thèse présentera également les perspectives de trois résultats majeurs de la bioconversion intégrée des biosolides en produits à valeur ajoutée qui s'accompagne de la réduction des solides et de l'élimination des contaminants dans les biosolides.

Rôle des microorganismes indigènes sur la valorisation des biosolides

• Les biosolides non stérilisés collectés à l'usine de traitement des eaux usées locale ont d'abord été congelés-dégelés, filtrés (filtre en fibre de verre de 0,7 µm) et préparés à la concentration requise de bouillie de biosolides. La concentration de la suspension de BS a été formulée en combinant BS avec des effluents d'eaux usées (WWE). Ensuite, cette suspension de BS a été caractérisée et digérée en aérobie pendant 28 jours.

• Au cours de l'atténuation naturelle des microbes, l'élimination totale des PhAC, pesticides, TSS et VSS était respectivement de 44 %, 10 %, 14,6 % et 18,6 %. Lorsque la suspension de BS est bio stimulée en ajoutant 1 g de mélange d'enzymes (GTB 2X) et/ou 5 g de source de carbone externe, l'élimination totale des PhACs, pesticides, TSS et VSS a été améliorée d'environ 7 à 18 %, 4 à 44 %, 1 -9% et 4-13% respectivement. Ces résultats élevés pourraient être attribués à

l'ajout de mélanges d'enzymes et/ou de sources de carbone, qui ont augmenté le métabolisme des microbes indigènes dans les biosolides.

• Les enzymes qui jouent le rôle majeur dans la dégradation des solides et les contaminants étaient présentes en abondance dans les biosolides. Les enzymes hydrolytiques telles que les protéases, l'amylase, les lipases et les phosphatases se sont révélées élevées dans les échantillons de suspension de BS prétraités aux enzymes, par rapport aux échantillons naturellement atténués ou à l'ajout séparé d'échantillons de source de carbone externe. D'autre part, l'activité laccase de l'enzyme ligninolytique était élevée dans les échantillons naturellement atténués par rapport à l'addition de glucose et/ou d'enzyme.

• L'ajout d'enzymes ainsi que la sécrétion d'enzymes extracellulaires par les microbes indigènes pendant la digestion aérobie ont amélioré le cocktail d'enzymes hydrolytiques dans les échantillons biostimulés. Au contraire, en raison de la biostimulation, il y a une plus grande disponibilité des glucides qui a induit un effet de moins d'activité laccase dans les échantillons biostimulés.

La plupart des enzymes oxydoréductrices obtenues au cours de cette thèse ont la capacité d'utiliser une gamme de substrats plus large et de l'oxygène moléculaire pour la catalyse, ce qui en fait un excellent choix pour l'assainissement d'une large gamme de contaminants. De même, les enzymes hydrolytiques, qui possèdent la capacité d'établir la vitesse limite globale des processus de digestion aérobie, et cette étape limitante implique la décomposition de gros substrats polymères tels que les glucides (cellulose, amidon, protéines) en petits composés de faible poids moléculaire (glucose , acides aminés, etc.), ce qui à son tour entraîne une réduction de la concentration en solides. Par conséquent, le cocktail d'enzymes obtenu au cours de cette étude pourrait être encourageant pour leur utilisation en biorestauration pour le traitement de contaminants préoccupants ainsi que pour la réduction des solides. Cependant, une étude plus détaillée est nécessaire afin de comprendre la pureté des enzymes libres obtenues et sa stabilité pour résister à des conditions opératoires vigoureuses.

La bioaugmentation assistée par prétraitement et son effet synergique avec les microbes indigènes sur la valorisation des biosolides

• L'impact de l'ajout de sources de carbone externes ou d'enzymes et de leurs combinaisons aux micro-organismes indigènes pendant la digestion aérobie des biosolides a amélioré l'élimination des contaminants dans une certaine mesure, mais la réduction des solides obtenus était relativement lente, à cause de la matrice de boue complexe.

• Les micro-organismes indigènes qui sont inefficaces ou relativement lents dans la biodégradation des contaminants présents dans l'environnement du site nécessitent la culture d'une nouvelle souche, ce qui améliore les performances des souches existantes ainsi que l'amélioration de l'élimination des contaminants.

• Ainsi, la bioaugmentation de *B. subtilis* en suspension de BS sans aucune biostimulation a été examinée, l'élimination totale des PhACs, pesticides et TSS était de 52 %, 50 % et 12 % ont été atteints.

• Du point de vue de la bioaugmentation et de l'atténuation naturelle, il a été conclu que les substances polymériques extracellulaires produites par les microbes pendant la digestion, se sont combinées pour former des flocs de boues, ce qui a réduit le taux de dégradation. Même si les enzymes produites par les microbes/l'ajout d'enzymes ne peuvent pas pénétrer dans la matrice de boues, ce qui a eu un impact sur la mauvaise réduction des solides et l'élimination moindre des contaminants.

• Afin d'améliorer la valorisation, le BS slurry est soumis à quatre stratégies de prétraitement différentes à savoir alcalin, ultrasonication, lyophilisation et traitement enzymatique. Tous les prétraitements ont un impact efficace sur la désintégration des boues, à l'exception du prétraitement enzymatique qui est le moins efficace/impact négligeable sur la désintégration.

• Considérant tous les prétraitements, le prétraitement alcalin s'est avéré décisif avec une élimination totale des PhACs, pesticides et TSS de 63 %, 64 % et 8 % respectivement, suivi par les ultrasons, qui étaient de 31 %, 34 % et 5 % pour le total des PhACs, pesticides et réduction du TSS.

• Mais le prétraitement alcalin a considérablement affecté les activités enzymatiques par rapport à d'autres prétraitements. Des concentrations élevées de sel qui augmentent la pression de turgescence dans l'environnement peuvent être une cause de ce changement drastique.

• Les suspensions de BS prétraitées ont été inoculées avec la souche *B. subtilis*. Compte tenu de l'élimination des contaminants, la bioaugmentation de *B. subtilis* assistée par prétraitement enzymatique s'est avérée efficace avec une élimination totale des PhACs et des pesticides de 89 % et 99 % respectivement. Cependant, du point de vue de la réduction solide, la bioaugmentation de B. subtilis assistée par prétraitement lyophilisé a entraîné une élimination maximale des TSS de 46% et elle a également 82% et 99% de l'élimination totale des PhACs et des pesticides respectivement. Dans l'ensemble, la bioaugmentation a amélioré l'élimination totale des PhACs, des pesticides et des TSS d'environ 21 à 80 %, 22 à 76 %, 8 à 54 % par rapport au prétraitement avant la bioaugmentation respectivement.

En dehors du cocktail enzymatique récupéré, les biosolides qui restent inutilisés avec la présence de *B. subtilis* obtenus au cours de cette étude peuvent être une bonne option viable pour une application terrestre en raison de son potentiel comme agent de lutte biologique. Ce qui est non pathogène et possède également une activité antimicrobienne comme avantage supplémentaire. Cependant, son impact sur d'autres communautés microbiennes qui incluent également des agents pathogènes et sa capacité potentielle en tant qu'agent de biocontrôle et activité antimicrobienne n'ont pas été étudiés, donc une étude métagénomique approfondie ainsi que sa capacité potentielle en tant que biocontrôle ainsi que sa nature antimicrobienne seront étudiées à l'avenir pour leur l'utilisation aux applications liées aux terres.

Enzymes d'intérêt de la bioaugmentation fongique des biosolides utilisées pour l'assainissement de l'eau synthétique

• Des tests de dépistage initiaux ont été effectués sur un échantillon de BS cultivé sur A. *niger.* L'effet de la concentration en BS, du temps de fermentation, du pH initial de la suspension, des caractéristiques de l'inoculum comme le type, l'âge et la taille de l'inoculum, ainsi que l'effet de la biostimulation en ajoutant des composants de milieu synthétique optimisés (saccharose, peptone, carbonate de calcium et sulfate de magnésium) ont été étudié sur des spores *d'A. niger* cultivées avec 5% (w/v) à des concentrations variables pendant 48h.

• D'après les résultats d'optimisation d'un échantillon de BS séché stérilisé à 25 % (p/v), un pH d'environ 5,0, une agitation à 150 tr/min, l'ajout de carbonate de calcium ajouté seul et une température à 30 °C ont donné l'activité GOD la plus élevée. L'enzyme GOD produite subira une expérience de sonication pendant 5 à 10 minutes et l'activité a été évaluée.

• L'action biocatalytique de GOD oxyde le glucose en gluconolactone, conduit à la génération in situ d'acide gluconique et de peroxyde d'hydrogène H2O2 par hydrolyse. Une concentration maximale de H2O2 de 216 ppm a été atteinte après 90 min de réaction lorsqu'environ 42 % du glucose 0,55 M a été oxydé avec 1000 U/L de GOD. Le H2O2 produit pourrait être davantage utilisé pour un processus d'oxydation avancé basé sur le bio-Fenton. Contrairement au processus de fenton classique, la réaction de bio-fenton par la médiation de DIEU annule l'exigence de pH acide (3,0).

• L'effet du ratio H2O2:FeSO4 de l'oxydation Bio-Fenton pour éliminer les composés pharmaceutiquement actifs a été étudié et un ratio H2O2:FeSO4 de 50:1 a montré une élimination comparativement plus élevée que 1:1. Bien qu'un rapport optimal concluant n'ait pas été établi, car le rapport réalisable pour différents composés pharmaceutiques varie.

• Jusqu'à 15 composés pharmaceutiquement actifs pourraient être partiellement éliminés, mais seulement moins de 50 % d'élimination pourraient être atteints. Cela peut être attribué à la conversion incomplète du glucose en GOD, au contaminant cible organique multiple et à la nature non sélective du radical hydroxyle.

La capacité de l'enzyme GOD à éliminer les PhACs dans l'eau synthétique a donné des résultats prometteurs, à l'avenir, elle peut être utilisée dans les effluents d'eaux usées. Cependant, ces résultats ne peuvent pas être appliqués au scénario en temps réel car le constituant des eaux usées varie également en fonction du fonctionnement unitaire des industries respectives. Ainsi, les paramètres de processus tels que l'effet du pH, de la température, de la concentration en enzymes, du temps et le rôle des médiateurs (par exemple O2 et H2O2) sur l'action enzymatique peuvent être considérés pour augmenter l'action des enzymes sur le processus de traitement seront étudiés à l'avenir. pour la biorestauration du site liée à l'environnement.

Dans l'ensemble, cette thèse offre une alternative efficace en proposant la valorisation des BS pour produire les produits à valeur ajoutée (cocktail d'enzymes hydrolytiques et oxydantes),

tandis que les bioproduits produits seront réutilisés pour la dépollution dans d'autres sites contaminés. Alors que l'humeur de l'univers évolue rapidement vers l'utilisation des déchets en ressources en raison de la durabilité environnementale. L'application de la réutilisation de la BS pour récupérer des bioproduits nécessite une approbation appropriée des autorités ainsi que des investissements en capital sont nécessaires pour présenter ces bioproduits sur le marché commercial. Comme cette politique de gestion des déchets, facilite le concept RRR (Réutilisation, Recyclage et Récupération) qui protège également l'environnement et l'écosystème du sol et contribue également à l'économie circulaire.

General conclusion and Future perspectives

If the process of production of hydrolytic and oxidative enzymes by various microorganisms (bacteria, fungi, etc.) in different nutrient media has been studied by several authors, the combination of municipal biosolids and wastewater as culture medium and the bacterial/fungal strain producing cocktail of oxidative and hydrolytic enzymes has not been widely used and is the cornerstone of this research project. As this thesis aims to develop an integrated bio approach allowing the reduction and treatment of biosolids while producing enzymes that can be valued for the treatment of water, so that the research approach will produce low-cost biocatalysts of industrial interest and achieve true, long-term, sustainability. The present work is an eco friendly process for converting solid waste into an intermediate substrate for the production of value added products. The thesis will also present the outlook of three major outcomes from the integrated bioconversion of biosolids to value added products which comes along with solids reduction and removal of contaminants in the biosolids.

Role of indigenous microorganisms on biosolids valorization

- The unsterilized biosolids collected from the local wastewater treatment plant were first freeze-thawed, filtered (0.7µm glass fibre filter), and prepared to the required concentration of biosolids slurry. BS slurry concentration was formulated by combining BS with wastewater effluent (WWE). Then, this BS slurry was characterised and aerobically digested for 28 days.
- During natural attenuation of microbes, the total PhACs, pesticides, TSS and VSS removal were 44%, 10%, 14.6% and 18.6% respectively. When BS slurry is bio

stimulated by adding 1g of enzyme mixture (GTB 2X) and /or 5g of external carbon source, the total PhACs, pesticides, TSS and VSS removal were improved by about 7-18%, 4-44%, 1-9% and 4-13% respectively. These elevated results could be attributed to the addition of enzyme mixtures and/or carbon sources, which increased the metabolism of indigenous microbes in biosolids.

- Enzymes which play the major role in the degradation of solids and contaminants were present plenty in the biosolids. Hydrolytic enzymes such as proteases, amylase, lipases, and phosphatases were found high in enzyme pretreated BS slurry samples while compared to naturally attenuated samples or separate addition of external carbon source samples. On the other hand, ligninolytic enzyme laccase activity was high in naturally attenuated samples while comparing to glucose addition and/ or enzyme addition.

- Addition of enzyme as well as secretion of extracellular enzymes by the indigenous microbes during aerobic digestion improved the hydrolytic enzyme cocktail in bio stimulated samples . On contrary, because of biostimulation, there is a higher availability of carbohydrates which induced an effect of less laccase activity in bio stimulated samples.

Most of the oxidoreductive enzymes obtained during this thesis having the ability to utilize a broader substrate range and molecular oxygen for catalysis which makes them an excellent choice for the remediation of a wide range of contaminants. Similarly, hydrolytic enzymes, which possess the ability to establish the overall limiting rate of the aerobic digestion processes, and this rate limiting step involves the breakdown of large polymeric substrates such as carbohydrates (cellulose, starch, proteins) into small low molecular compounds (glucose, amino acids, etc.), which in turn results in reduction of solids concentration. Hence, cocktail of enzymes obtained during this study could be an encouraging one for their usage in bioremediation for the treatment of contaminants of concern as well as solids reduction. However, more detailed study

is required in order to understand the purity of obtained free enzymes and its stability to withstand vigorous operating conditions.

Pretreatment assisted bioaugmentation and its synergetic effect along with indigenous microbes on biosolids valorization

- Impact of adding external carbon sources or enzymes and their combinations to indigenous microorganisms during aerobic digestion of biosolids improved the contaminants removal to some extent but the obtained solids reduction were relatively slow, its because of the complex sludge matrix.
- Indigenous microorganisms which are ineffective or relatively slow in biodegradation of contaminants present in the environment site requires a cultivation of new strain, which improves the performance of the existing strains along with enhancement of contaminants removal.
- So, Bioaugmenting *B. subtilis* to BS slurry without any biostimulation were examined, the total PhACs, pesticides and TSS removal were 52% ,50% and 12% were achieved.
- From the bioaugmentation and natural attenuation perspective, it has been concluded that extracellular polymeric substances produced by the microbes during the digestion, combined together to form sludge floc, which has reduced the rate of degradation. Even thought the enzymes produced by the microbes/addition of enzyme can't able to penetrate the sludge matrix which impacted the poor solids reduction and lesser contaminants removal.
- In order to improve the valorization, BS slurry is subjected to four different pre-treatment strategies namely alkali, ultrasonication, freeze-drying and enzymatic treatment. All PT have effective impact on disintegrating sludge expect enzymatic PT which is least effective/ negligible impact on disintegration.

- Considering all PT, alkali PT found to be decisive one with total PhACs, pesticides and TSS removal of 63%, 64% and 8% respectively, followed by ultrasonication, which were 31%,34% and 5% for total PhACs, pesticides and TSS reduction.
- But alkali PT have drastically affects enzyme activities while compared to other PT. High salt concentrations which increases the turgor pressure to the environment can be a cause for this drastic change.
- The pre-treated BS slurries were inoculated with *B. subtilis* strain. Considering contaminants removal, enzymatic PT assisted *B. subtilis* bioaugmentation has found to be effective with total PhACs and pesticides removal of 89% and 99% respectively. However, on solid reduction perspective, freeze-dried PT assisted *B. subtilis* bioaugmentation incurred maximum TSS removal of 46% and also it has 82% and 99% of total PhACs and pesticides removal respectively. Overall, bioaugmentation improved the total PhACs, pesticides and TSS removal by about 21-80%, 22-76%, 8-54% while compared to PT before bioaugmentation respectively.

Apart from enzyme cocktail recovered, biosolids which remains unutilized with presence of *B.subtilis* obtained during this study can be a good viable option to land related application because of its potential as biocontrol agent. Which is a non-pathogen and also possesses an antimicrobial activity as an additional advantage. However, its impact over other microbial communities which also includes pathogens and its potential ability as biocontrol agent and antimicrobial activity were not studied, so in depth metagenomic study along with its potential ability as biocontrol as well as antimicrobial nature will be studied in future for their usage to land related applications.

Enzymes of interest from fungal bioaugmentation of biosolids utilized for the remediation of synthetic water

- Initial screening tests were performed on BS sample grown on *A. niger*. The effect of BS concentration, fermentation time, initial pH of the slurry, inoculum characteristics like

inoculum type, age and size, as well as the effect of biostimulation by adding optimized synthetic media components (sucrose, peptone, calcium carbonate and magnesium sulfate) were studied on spores of *A. niger* cultured with 5% (w/v) at varying concentrations for 48hrs.

- From the optimization results of 25% (dw/v) sterilized dried BS sample, pH around 5.0, agitation at 150 rpm, addition of calcium carbonate added alone and temperature at 30° C were yielded the highest GOD activity. Yielded GOD enzyme will undergone sonication experiment for 5-10 mins and the activity was assessed.
- The biocatalytic action of GOD oxidizes glucose to gluconolactone, leads to the in-situ generation of gluconic acid and hydrogen peroxide H2O2 by hydrolyzation. Maximum concentration of H2O2 of 216 ppm was reached after 90 min of reaction when around 42% of 0.55 M glucose was oxidized with 1000 U/L GOD. The H2O2 produced could be further utilized for bio-Fenton based advanced oxidation process. Unlike the classical fenton process, bio-fenton reaction by GOD mediation negates the acidic pH (3.0) requirement.
- The effect of H2O2:FeSO4 ratio Bio-Fenton oxidation to remove pharmaceutically active compounds was studied and a H2O2:FeSO4 ratio of 50:1 showed comparatively higher removal than 1:1. Although a conclusive optimum ratio was not established as the feasible ratio for different pharmaceutical compounds varies.
- Up to 15 pharmaceutically active compounds could be partially removed but only less than 50% removal could be achieved. This may be attributed to the incomplete conversion of glucose to GOD, multiple organic target contaminant and non-selective nature of the hydroxyl radical.

GOD enzyme ability over removal of PhACs in synthetic water has shown promising results, in future it can be utilized in wastewater effluent. However, these findings cannot be applied to the real-time scenario as the constituent of wastewater also varies according to the unit operation of the respective industries. So, the process parameters such the effect of pH, temperature, enzyme concentration, time and the role of mediators (e.g. O_2 and H_2O_2) on the enzyme action can be

considered to increase action of the enzymes on the treatment process will be studied in future for environmental related site bioremediation.

Overall, this thesis provides an effective alternative by offering the valorization of BS to produce the value added products (cocktail of hydrolytic and oxidative enzymes), while the produced bioproducts will be reused for the remediation in other contaminated site. As the mood of universe is changing swiftly towards the utilization of waste to resource because of environmental sustainability. The application of reusing BS to recover bioproducts needs an appropriate approval from the authorities as well as the capital investments are needed to showcase this bioproducts in the commercial market. As this waste management policy, facilitates the RRR (Reuse, Recycling and Recovery) concept which also protects the environment and soil ecosystem and also contributing the circular economy.

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ANNEXURES

Annexe 1: Informations supplémentaires - Chapitre 4

Effet des microorganismes indigènes et de leur enrichissement sur la valorisation des biosolides

Tableau 4- Tested PhACs, herbicide, fungicide and insecticide compounds for their presence in BS.

Contaminant		**Molecular weight (g/mol)**	**Log k_{ow}**	**Pharmacology**
Fungicide	Carbendazim	191.19	1.52	Fungicide
	Thiabendazole	201.25	2.47	Fungicide, Parasiticide
	Pyrimethanil	199.26	2.84	Fungicide
	Kresoxim methyl	313.3	3.4	Fungicide
	Pyraclostrobin	387.8	3.99	Fungicide
	Trifloxystrobin	408.37	4.5	Fungicide
	Boscalid	343.2	2.96	Fungicide
	Iprodione	330.16	3	Fungicide, nematicide
	Fludioxonil	248.18	4.12	Fungicide
Herbicide	Diuron	233.09	2.68	Herbicide
	Simazine	201.65	2.18	Herbicide
	Isoproturon	206.3	2.87	Herbicide
	Chlortoluron	212.67	2.41	Herbicide
	Monolinuron	214.6	2.3	Herbicide, algaecide
	Atrazine	215.68	2.61	Herbicide
	Metoxuron	228.69	1.6	Herbicide
	Terbuthylazine	229.71	3.4	Herbicide
	Cyanazine	240.69	2.22	Herbicide
	Linuron	249.09	3.2	Herbicide
	Hexazinone	253.32	1.85	Herbicide

	Pendimethalin	281.31	5.2	Herbicide
	Metolachlor	283.79	3.13	Herbicide
	Imazethapyr	289.33	1.49	Herbicide
	Metobromuron	259.1	2.4	Herbicide
	Bentazon	240.28	2.34	Herbicide
Insecticide	Aldicarb	190.27	1.13	Insecticide
	Carbofuran	221.25	2.32	Insecticide
	Acetamiprid	222.67	0.8	Insecticide
	Carbaryl	201.22	2.36	Insecticide
	Dinotefuran	202.21	0.64	Insecticide
	Dimethoate	229.26	0.78	Insecticide, acaricide
	Omethoate	213.19	-0.74	Insecticide
	Bendiocarb	222.23	1.7	Insecticide
	Clothianidin	249.67	0.7	Insecticide
	Thiacloprid	252.72	1.26	Insecticide
	Imidacloprid	255.66	0.57	Insecticide
	Nitenpyram	270.72	-0.66	Insecticide
	Thiamethoxam	291.71	-0.13	Insecticide
	Parathion	291.3	3.83	Insecticide
	Diazinon	304.35	3.3	Insecticide
	Azinphos-methyl	317.32	2.75	Insecticide
	Phosmet	317.3	2.95	Insecticide
	Chlorpyrifos	350.59	5	Insecticide
	Malathion	330.4	2.36	Insecticide
	Coumaphos	362.77	4.13	Insecticide
	Chlorfenvinphos	359.6	3.81	Insecticide, acaricide
	Permethrin	391.28	6.5	Insecticide
	Spinosad	732	2.8	Insecticide
	Piperonyl butoxide	338.43	4.75	Synergist component of pesticide

Pharma-ceuticals	Naproxen	230.26	3.18	Analgesic, Antipyretic
	Caffeine	194.19	-0.07	CNS stimulant
	Carbamazepine	236.27	2.45	Anticonvulsant, Analgesic
	Mefenamic acid	241.29	3.75	Analgesic, Antipyretic, Anti-inflammatory
	Ketoprofen	254.29	3.12	Analgesic, Anti-inflammatory
	Trimethoprim	290.32	0.91	Antibacterial
	Fenofibrate	360.83	5.19	Lipid regulator
	Ibuprofen	206.28	3.97	NSAID
	Ifofsamide	261.08	0.86	Chemotherapeutic agent
	Cyclophosphamide	261.08	0.63	Antineoplastic, immunosuppresive
	Atenolol	266.34	0.16	Beta-blocker
	Ciprofloxacin	331.34	0.28	Antibiotics
	Indomethacin	357.78	0.91	NSAID
	Bezafibrate	361.82	3.97	Antilipemic
	Ofloxacin	361.36	-0.39	Antibiotic

Tableau 5- Biochemical compositional changes of biosolids due to indigenous microbe digestion and investigation of bio-catalysis and bio-stimulation as pretreatment strategies

Parameter	**Time (days)**	**Pretreatment**			
		T1	**T2**	**T3**	**T4**
Carbohydrates Concentration (mg/mL)	0	0.02±0.00	0.14±0.01	0.18±0.02	0.20±0.05
	2	0.19±0.02	0.50±0.00	0.54±0.02	0.67±0.02
	4	0.34±0.09	0.63±0.06	0.84±0.09	1.34±0.09
	7	0.67±0.08	0.74±0.14	1.22±0.01	1.46±0.06
	10	0.77±0.11	1.12±0.29	1.37±0.11	1.64±0.11
	14	0.81±0.09	1.36±0.09	1.53±0.03	1.80±0.14

	21	0.57±0.05	1.52±0.56	1.80±0.13	2.13±0.09
	28	0.44±0.00	0.72±0.12	2.02±0.39	2.36±0.23
Protein Concentration (mg/mL)	0	0.50±0.03	0.57±0.02	0.72±0.04	0.84±0.00
	2	0.59±0.05	0.64±0.05	0.79±0.05	0.89±0.05
	4	0.67±0.11	0.70±0.03	0.67±0.03	0.92±0.03
	7	0.52±0.09	0.47±0.02	0.63±0.00	0.77±0.10
	10	0.47±0.00	0.43±0.00	0.54±0.05	0.69±0.05
	14	0.42±0.06	0.39±0.06	0.50±0.01	0.66±0.03
	21	0.28±0.03	0.47±0.02	0.43±0.01	0.63±0.04
	28	0.21±0.03	0.59±0.14	0.39±0.02	0.67±0.03

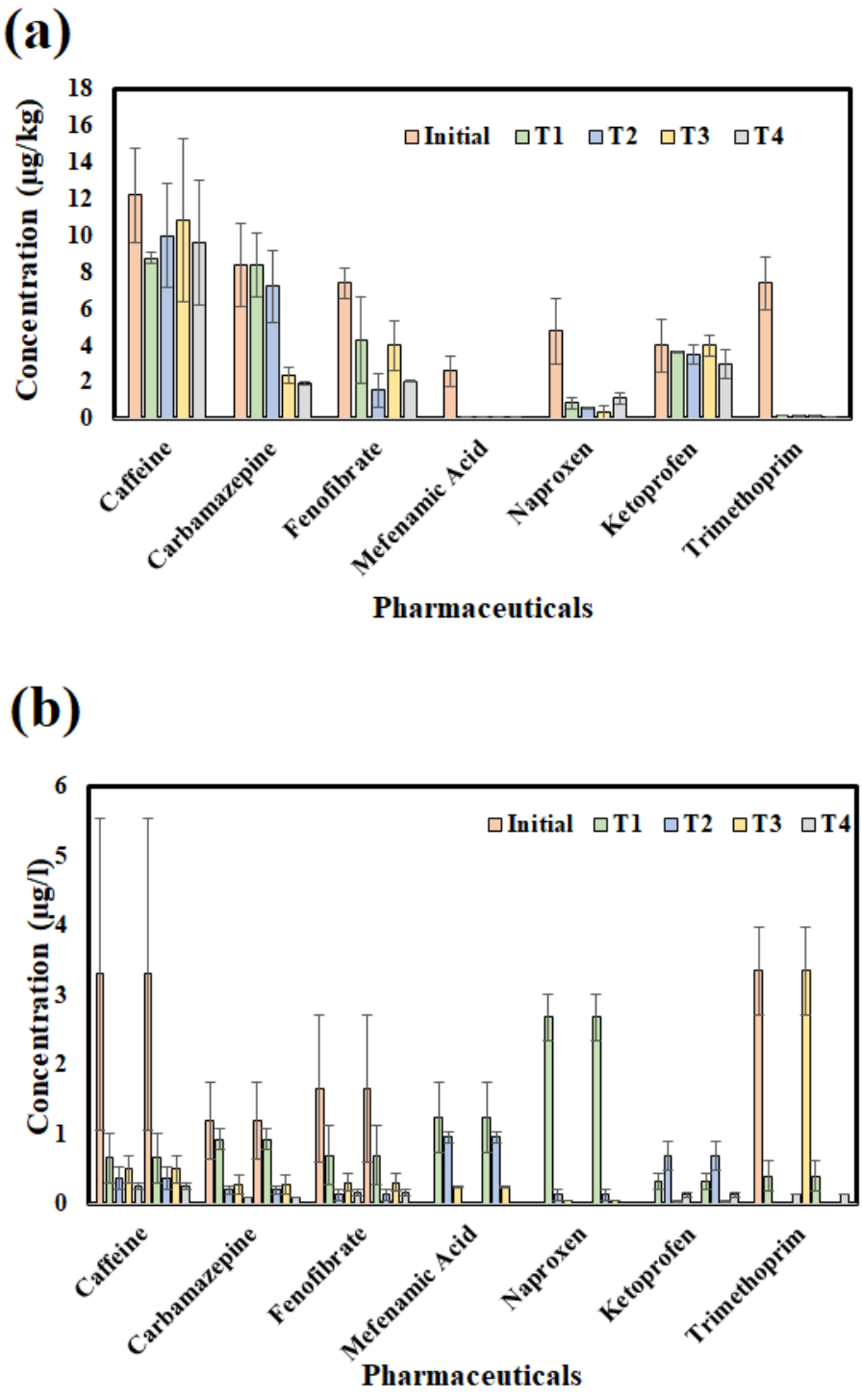


Figure 5- Concentration of pharmaceuticals in (a) solid, and (b) aqueous phase during digestion of biosolids slurry by various treatments (T1 – T4)

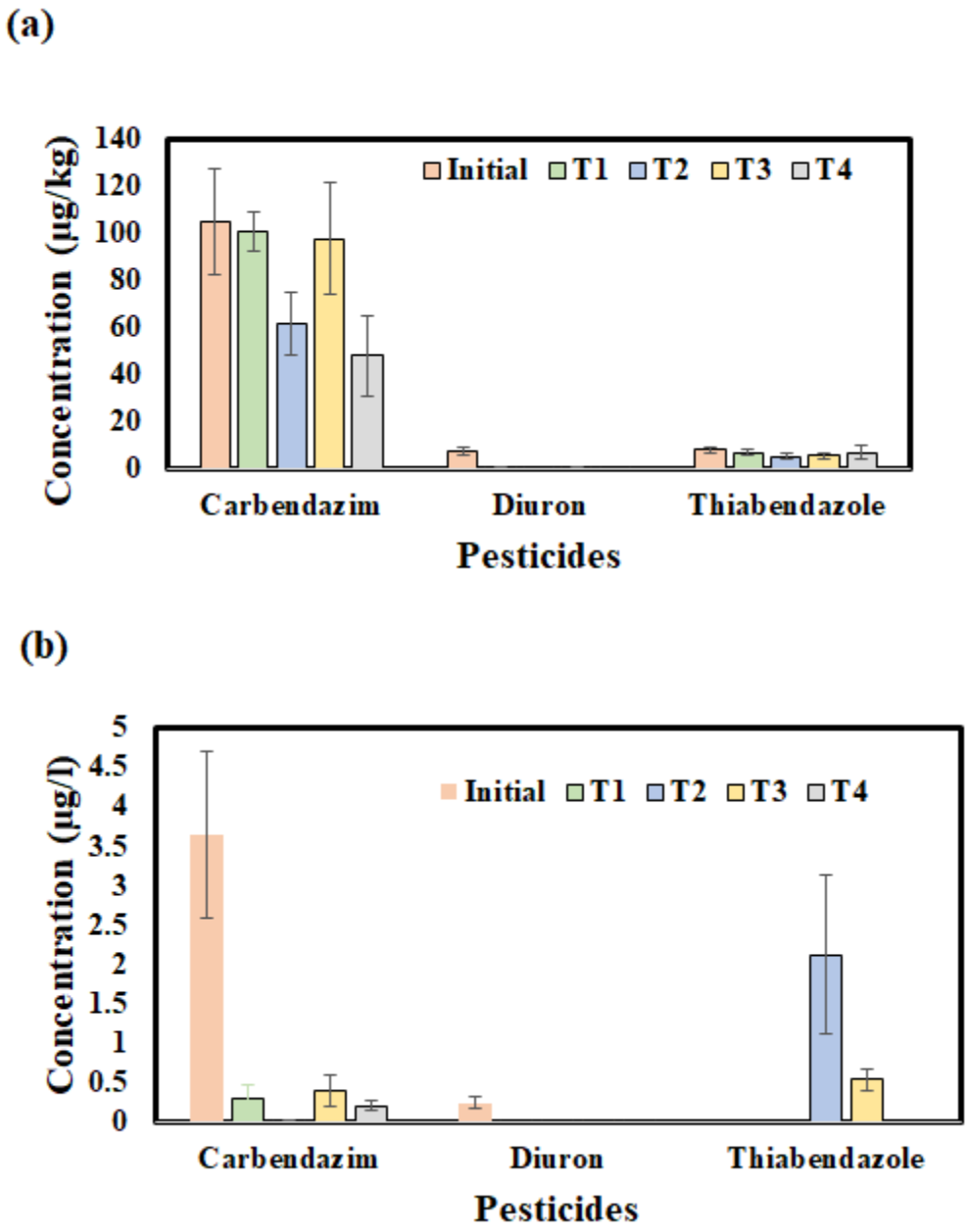


Figure 6- Concentration of pesticides in (a) solid and (b) aqueous phase during digestion of biosolids slurry by various treatments (T1 – T4)

Annexe 2: Informations supplémentaires - Chapitre 5

Évaluation de l'effet synergique de la bioaugmentation assistée par prétraitement avec les micro-organismes indigènes sur la valorisation des biosolides

Tableau 3- Biochemical compositional changes of biosolids due to bioaugmentation of *B. subtilis.*

Parameter	**Time**	**Bioaugmentation**			
	(days)	**Sonication**	**Freeze-dried**	**Enzymatic**	**Alkaline**
Protein (mg/g)	0	111.1 ± 22.9	45.5 ± 2.4	79.3 ± 1.0	164.3 ± 39.2
	7	26.2 ± 5.2	17.4 ± 1.8	67.4 ± 1.5	139.2 ± 51.4
	14	11.4 ± 0.1	13.6 ± 0.0	21.8 ± 0.9	189.3 ± 14.6
	21	25.8 ± 0.3	16.3 ± 0.1	12.5 ± 0.4	93.3 ± 24.4
	28	46.5 ± 1.8	10.1 ± 0.4	10.7 ± 0.2	106.3 ± 17.3
Sugar (mg/g)	0	1.3 ± 0.06	0.8 ± 0.01	0.92±0.04	3.4 ± 0.41
	7	0.6 ± 0.12	0.23 ± 0.01	1.0±0.07	1.9 ± 0.26
	14	0.44 ± 0.02	0.27 ± 0.12	0.9±0.05	2.3± 1.13
	21	0.47 ± 0.0	0.19 ± 0.08	0.25±0.07	-
	28	0.31 ± 0.13	0.11 ± 0.05	0.08±0.00	-

Tableau 4- Comparative assessment of various pretreatment methods and/or subsequent aerobic digestion on solids removal

Biosolids/ Sludge	Nature	Pre-treatment	Microorganism	Time	Solid Removal (%)	References
Municipal Biosolids from Quebec WWTP, Canada	Non-sterile	-	*B. subtilis*	28 days	12	Present Study
	Non-sterile	Ultrasonication		28 days	13.5	
	Non-sterile	Freeze drying		28 days	46	
	Non-sterile	Enzymatic		28 days	17.6	
	Non-sterile	Alkali		28 days	33.6	
Domestic wastewater treatment plant sludge, Malaysia	Non-Sterile		*A. niger* and *Penicilliu m corylophil um*	8 days	98.8	[296]
Domestic wastewater treatment plant sludge, Malaysia	Non-Sterile		*A. niger* and *P.coryloph ilum*	10 days	98	[297]
Domestic wastewater treatment plant sludge, Malaysia	Non-Sterile		*Mucor hiemalis* Wehmer	5 days	96	[298]

Municipal wastewater sludge, Quebec	Sterile	Sterilization	*Penicilliu m expansum* BS30	5 Days	40 - 60 %	[299]
	Non- sterile			3 Days	44 .7	
Waste activated sludge from oil refinery plant, Korea	Non-sterile	Ultrasonicati on	*Bacillus stearother mophilus*	3 days	47 .6	[218]
	Non-sterile	Ball mill		3 days	68 .1	
	Non-sterile	Thermal		3 days	66 .9	
	Non-sterile	Alkali		3 days	53 .6	
Anaerobic sludge from South Africa	Non-sterile	Enzymatic		5 days	80	[214]
Activated sludge from China	Non-sterile	Enzymatic		2 hours	0. 01	[239]
Waste activated sludge, India	Non-sterile		*B. jerish 3 and B. jerish 4*	20 days	48 .5	[120]
Waste activated sludge, China	Non-sterile	Ultrasonicati on		10.5 days	42 .7	[57]
Activated sludge from France	Non-sterile	Ultrasonicati on		50 days	45 .3	[300]
Activated	Non-sterile	Ozonation		50 days	71	
sludge from	Non-sterile	Ultrasonicati		50 days	76	[79]

France		on				
Waste activated sludge from beer production plant, China	Non-Sterile	Microwave-Alkaline		30 days	63 %	[301]
Waste activated sludge, India	Non-sterile	Ultrasonication	*B. jerish 3 and B. jerish 4*	20 days	39	[64]
Waste Activated sludge	Non-sterile		*B. lichenformis*	30 days	23	[124]
Waste Activated sludge	Non-sterile		*B. lichenformis*	20 days	59	[302]

Tableau 5- Comparative assessment of various pretreatment methods and/or subsequent aerobic digestion on TrOCs removal

Biosolids/ Sludge	**Nature**	**Pre-treatment**	**Microorganism**	**Time**	**Trace Organic Contaminants removal (%)**	**References**
Municipal Biosolids from Quebec WWTP, Canada	Non-sterile	-	*B. subtilis*	28 days	Pharmaceuticals – Pesticides - Pharmaceuticals – 60 Pesticides - 78 Pharmaceuticals – 82 Pesticides - 99 Pharmaceuticals –	Present Study
	Non-sterile	Ultrasonication		28 days		
	Non-sterile	Freeze drying		28 days		
	Non-sterile	Enzymatic		28 days		

					89	
					Pesticides - 99	
					Pharmaceuticals –	
	Non-sterile	Alkali		28 days	84	
					Pesticides - 86	
	Sterile	Sterilization	*T. versicolor*	42 days	Naproxen-47%	
					Carbamazephine-50%	[222]
					Pharmaceuticals	
	Sterile	Sterilization	*T. versicolor*	42 days	(83%),	
					Polychlorinated biphenyls	[133]
					(28.2%)	
	Sterile	Sterilization	*T. versicolor*	42 days	Sulfonamide-100%	[134]
El Prat de Llobregat WWTP, Barcelona, Spain					Pharmaceuticals (26-100%).	
	Sterile	Sterilization	*T. versicolor*	26 days	UV filters (22-100%)	[95]
					Brominated flame retardants (16-53%)	
	Non-Sterile		*T. versicolor*	42 days	Pharmaceuticals (56-100%)	[132]
	Sterile	Sterilization	*T. versicolor*	42 days	Pharmaceuticals (60-100%)	
					Pharmaceuticals	
	Non-sterile		*T. versicolor*	42 days	(86%),	
					Brominated-flame-retardants	[122]

					(81%) and UV filters (80%)	
	Non-sterile		*T. versicolor*	42 days	Pharmaceuticals (66%),	[121]
Activated sludge from Japan	Sterile	Sterilization		7 days	Pharmaceuticals (18-99.8%)	[303]

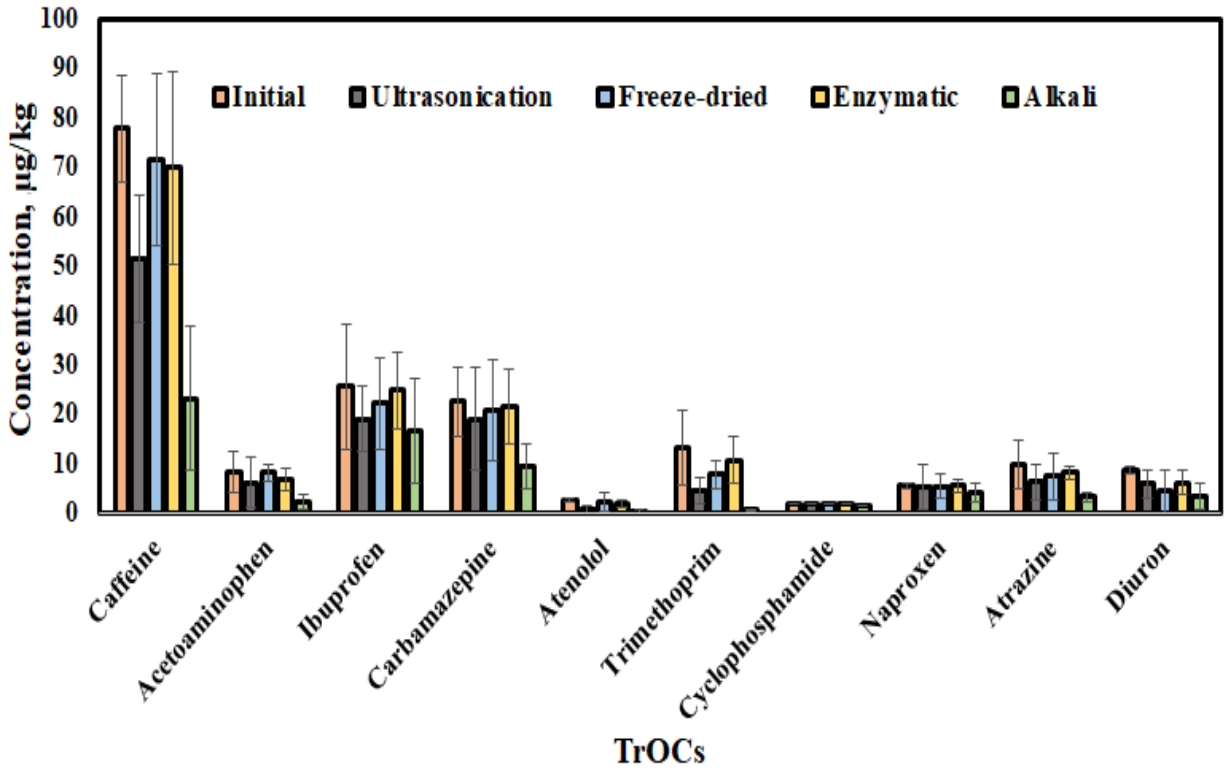


Figure 7- TrOCs concentration after pretreatments in solid phase (Initial: Caffeine-77.2±10.8 µg/kg, Acetaminophen-8.2±4.2 µg/kg, Ibuprofen-25.6±12.7 µg/kg, Carbamazepine-22.6±7.0 µg/kg, Atenolol-2.6±0.5 µg/kg, Diuron-8.8±0.8 µg/kg, Atrazine-10±4.9 µg/kg, Naproxen-5.6±0.5 µg/kg, Cyclophosphamide-2.0±0.0 µg/kg, Trimethoprim-13.4±7.6 µg/kg).

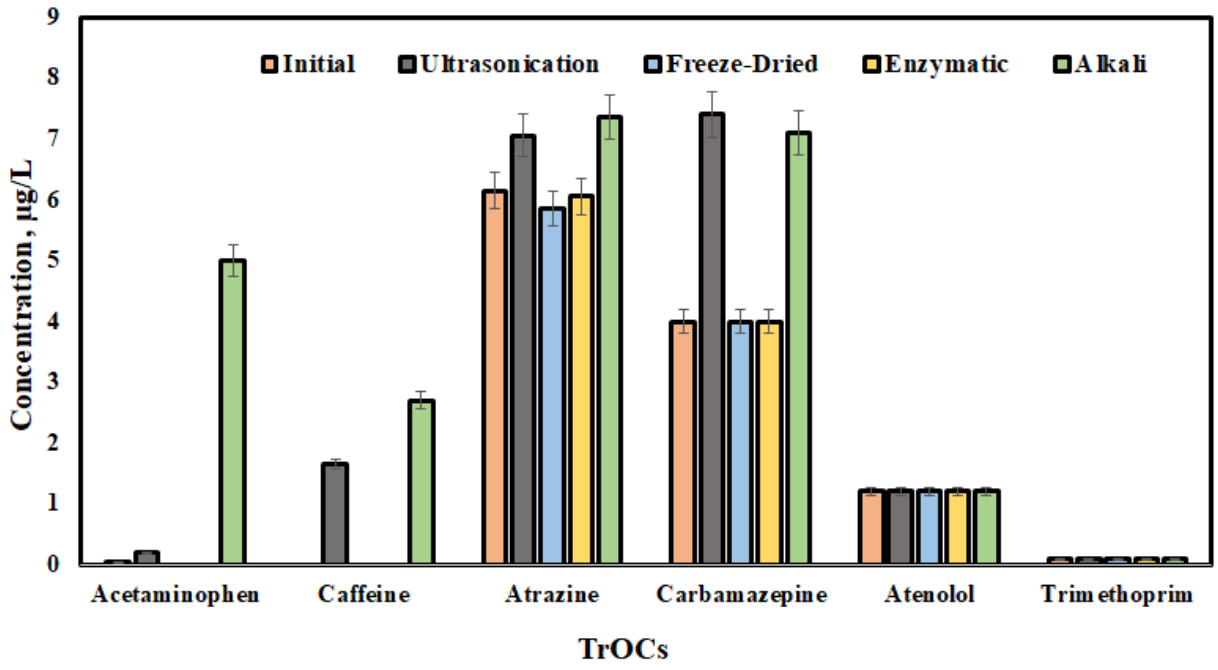


Figure 8- TrOCs concentration after pretreatments in aqueous phase (Initial: Caffeine-ND, Acetaminophen-0.05±0.05 µg/L, Carbamazepine-4±2 µg/L, Atenolol-1.2±1.2 µg/L, Atrazine-6.1±0.2 µg/L,Trimethoprim-0.1±0.1 µg/L).

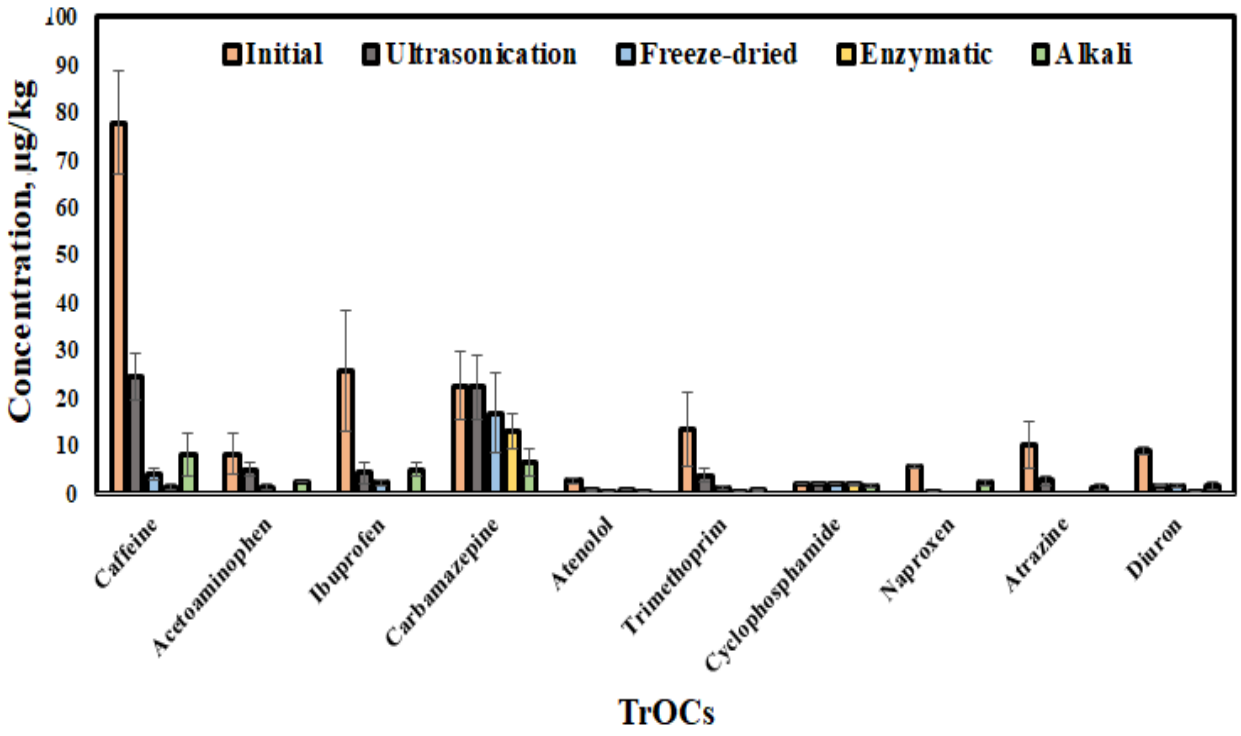


Figure 9- TrOCs concentration after bioaugmentation of *B. subtilis* with pre-treated biosolids after 28 days in solid phase. (Initial: Caffeine-77.2±10.8 µg/kg, Acetaminophen-8.2±4.2 µg/kg, Ibuprofen-25.6±12.7 µg/kg, Carbamazepine-22.6±7.0 µg/kg, Atenolol-2.6±0.5 µg/kg, Diuron-8.8±0.8 µg/kg, Atrazine-10±4.9 µg/kg, Naproxen-5.6±0.5 µg/kg, Cyclophosphamide-2.0±0.0 µg/kg, Trimethoprim-13.4±7.6 µg/kg).

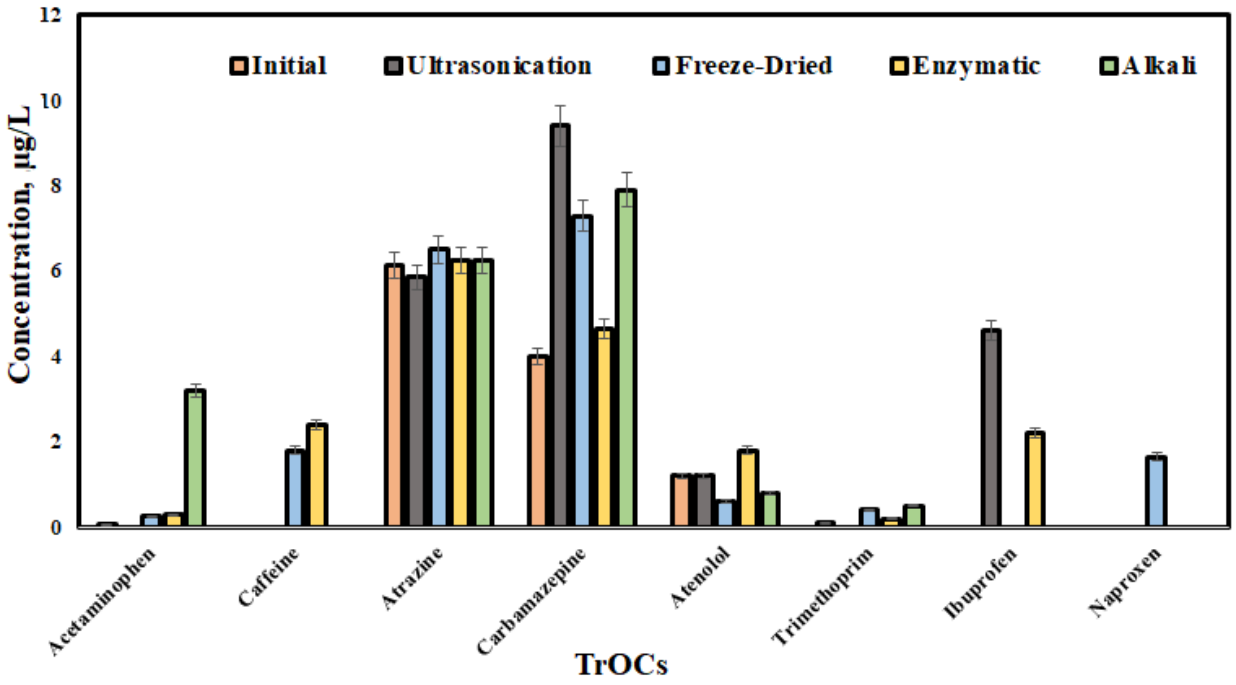


Figure 10- TrOCs concentration after bioaugmentation of *B. subtilis* with pre-treated biosolids after 28 days in aqueous phase. (Initial: Caffeine-ND, Acetaminophen-0.05±0.05 µg/L, Ibuprofen-ND, Carbamazepine-4±2 µg/L, Atenolol-1.2±1.2 µg/L, Atrazine-6.1±0.2 µg/L, Naproxen-ND, Trimethoprim-0.1±0.1 µg/L).

Annexe 3: Informations supplémentaires - Chapitre 6

Enzymes d'intérêt produites à partir de biosolides augmentés de champignons et leur utilisation pour le traitement des problèmes environnementaux

Tableau 1- Characterization of 50% unsterilized BS bioslurry prepared by mixing unsterilized BS and WWE

Bioslurry		
S. No.	**Characteristics**	**Values**
1	Total proteins (mg/g)	10.10 ± 0.66
2	Total reducing sugar (mg/g)	0.490 ± 0.03
3	Total Carbon (mg/g)	298.3 ± 3.07
4	Total Hydrogen (mg/g)	47.6 ± 0.08
5	Total Nitrogen (mg/g)	45.2 ± 0.14
6	Total Phosphorus (mg/g)	2.13 ± 0.11
7	TSS (mg/ml)	70.7 ± 2.14
8	TDS (mg/ml)	5.5 ± 0.3
9	C: N ratio	6.59
10	pH	6.8
11	Enzyme status	Lipase 90 U/L; Phosphatase 93 U/L; Dehydrogenase 58 U/L; Esterase of 40 U/L
Dried BS		
12	Metal ions (mg/g)	90

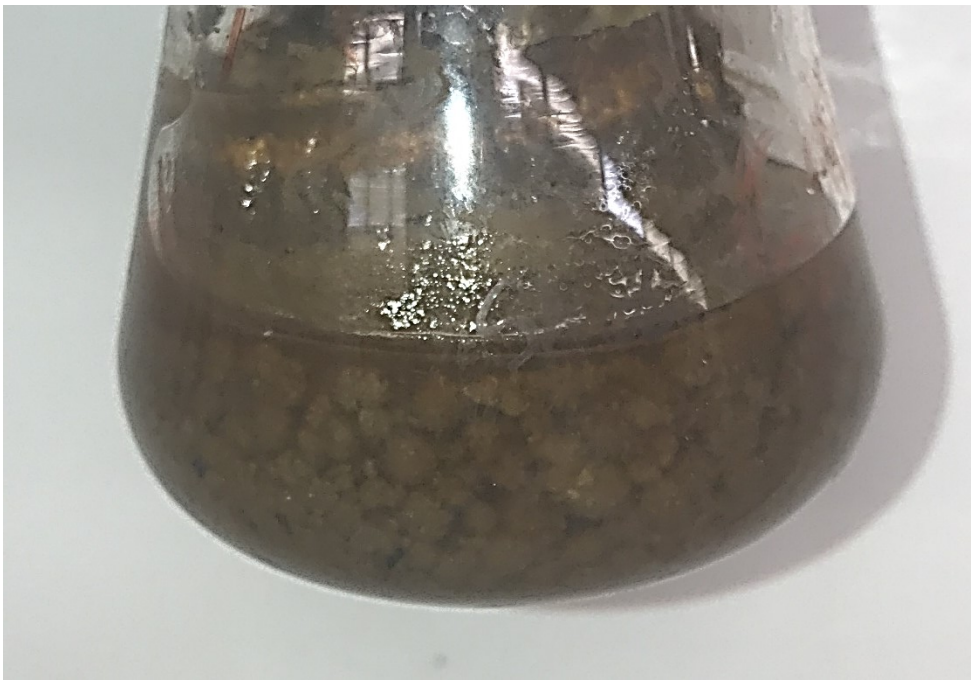
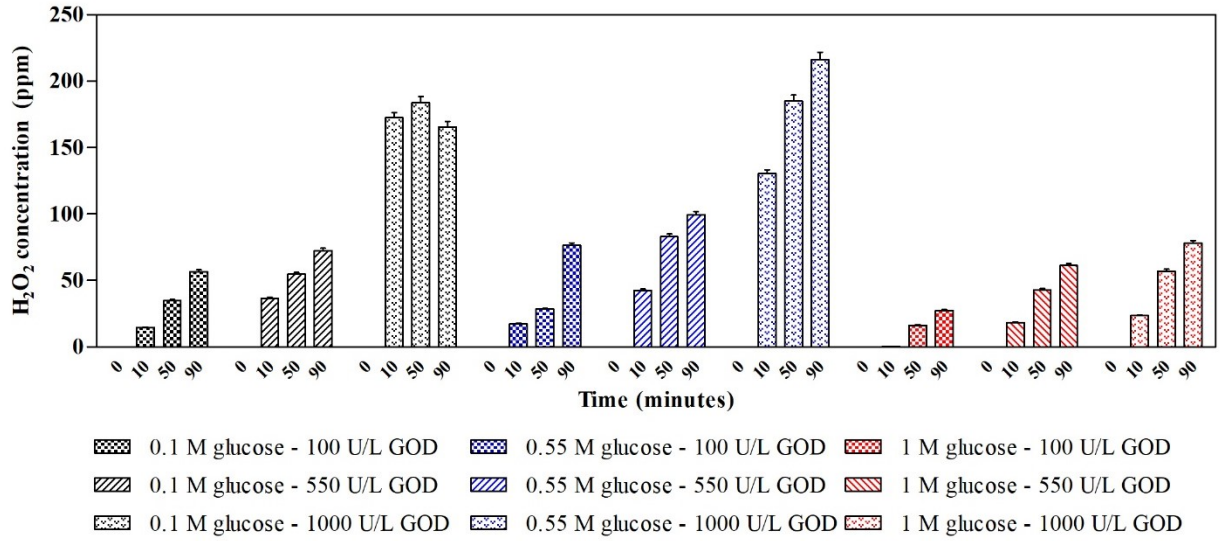


Figure 10- Agglomeration of *Aspergillus niger* in 25% (dw/v) BS slurry

Production media prepared by mixing DS-BS in WWE and inoculated with 5% of 2-day inoculum cultured in GYM media.

(a)



(b)

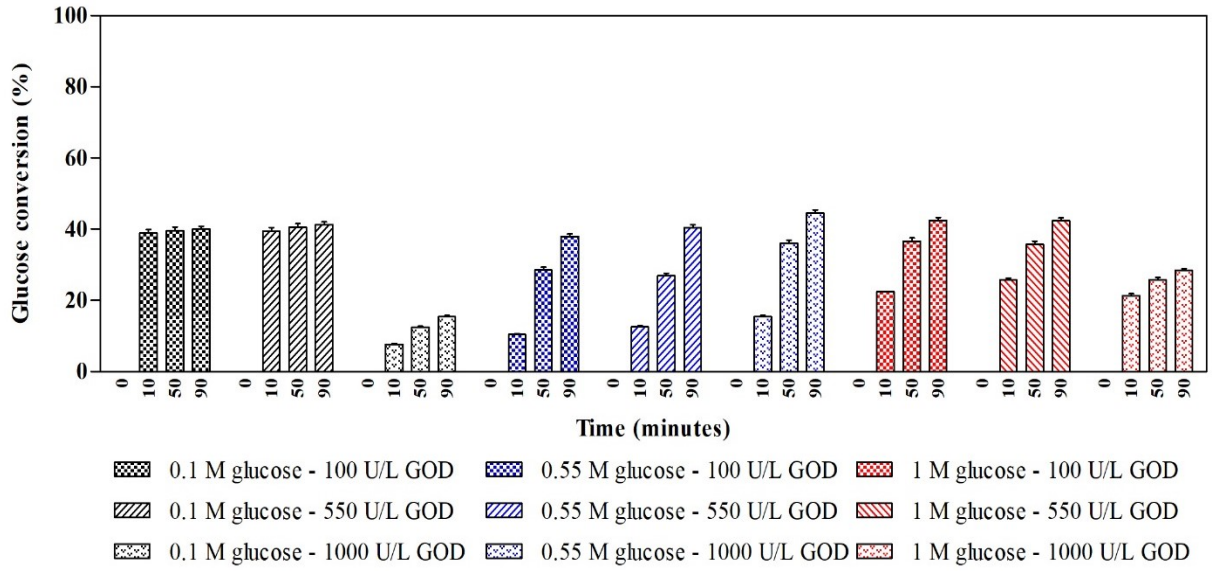


Figure 11- Glucose oxidation using BS-based GOD. (a) Glucose conversion obtained with different concentrations of glucose with varying activities of GOD; (b) Hydrogen peroxide concentration obtained with different concentrations of glucose with varying activities of GOD.

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