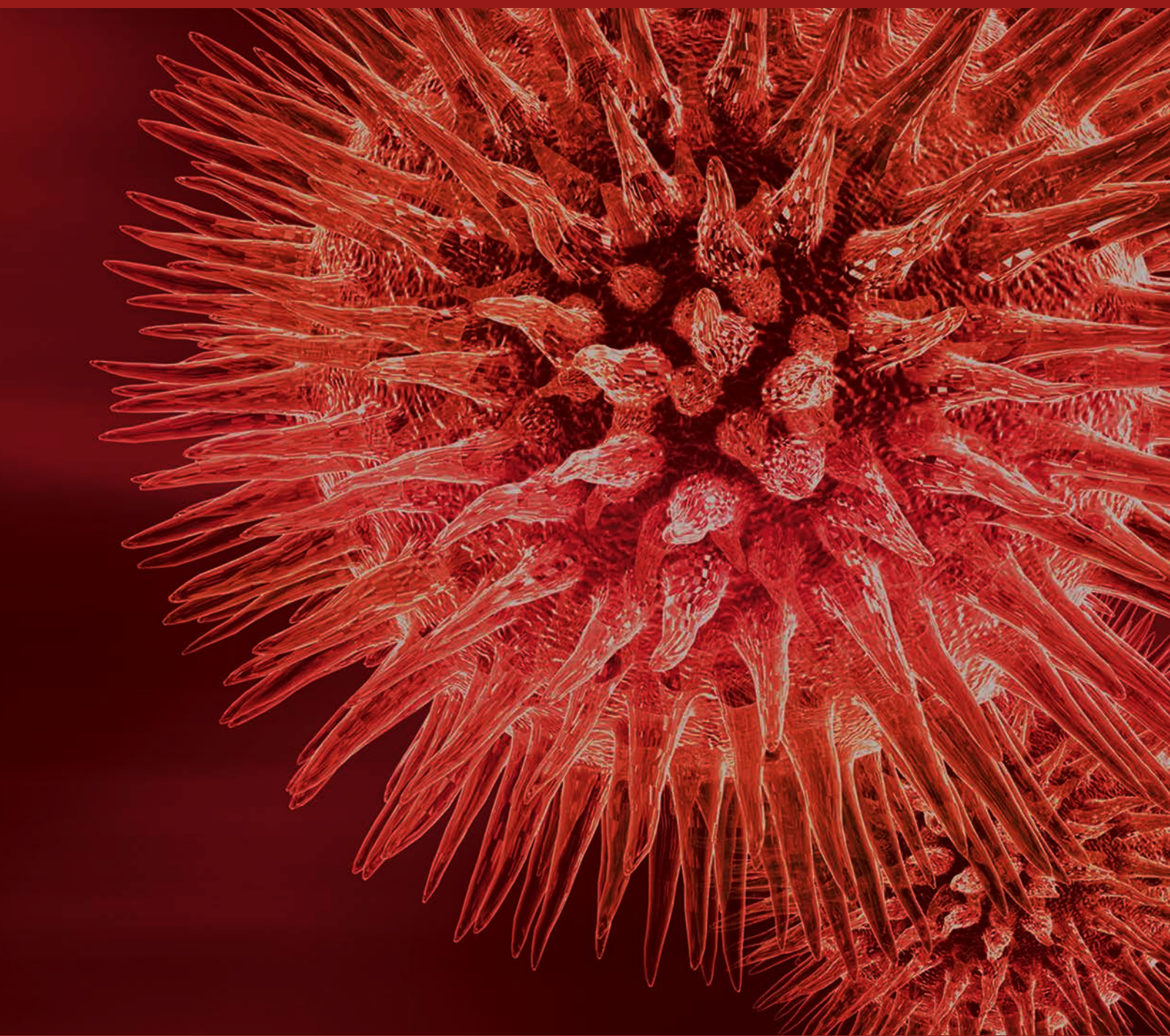
BioMed Research International

Frontiers in the Expansion of Bioproducts

Guest Editors: Junio Cota, Joachim Venus, Zaira B. Hoffmam, and Lucas F. Ribeiro



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Editorial

Frontiers in the Expansion of Bioproducts

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Bioproducts and biobased technologies are increasingly taking on outstanding position in the global market. Although there is debate on the longevity of the oil reserves, the global warming and its devastating consequences cannot be denied and probably will put an end to the fossil fuels era and start a new one with dependence on renewable energy. In December 2015, in an unprecedented agreement, 195 nations (including all of the top 10 emitting countries) committed to The Paris Agreement to hold "the increase in the global average temperature to well below 2°C above preindustrial levels and to pursue efforts to limit the temperature increase to 1.5°C above pre-industrial levels, recognizing that this would significantly reduce the risks and impacts of climate change" [1], which presumably would promote a significant increase in investments in green technologies.

Renewable raw materials have already been used to manufacture a wide variety of bioproducts in biorefineries worldwide, which may be divided into three large groups: biochemicals, biomaterials, and energy fuels. The concept of a self-sustainable biorefinery would ideally share manufacturing processes to produce bulk chemicals and high market value biobased chemicals at low cost, using cheap substrate and/or processes. However, industrial processes are mostly far from that model, since a lot of bottlenecks arise from each particular bioprocess. Nevertheless, 50 million tonnes of bioproducts were generated in 2012, including nonfood starch, cellulose fibres and cellulose derivatives, tall oils, fatty acids, and fermentation products such as alcohols, ketones, esters, organic acids, biopolymers, enzymes, amino acids, and vitamins [2]. Currently, the production of biobased commodity is commonly impaired by the competitive low price of crude oil, which constitutes a barrier to justify production costs. In a broader perspective, worldwide exportations of products in agriculture and forestry, food, bioenergy, biotechnology, and green chemistry were estimated to be about US $2 trillion in 2014, accounting for 13% of world trade [3]. The global renewable chemicals market reached a remarkable size of US $49 billion in 2015 and is forecasted to increase to US $84.3 billion by 2020. Just the global market for fermentation derived fine chemicals saw a revenue of US $16 billion in 2009, this sector being boosted by modern biotechnology that allows industry to improve the economics of new and old fermentation products.

In this context, this special issue introduces new concepts and trends regarding biobased processes and consists of four original researches and one review article. In one article, I. Baumann and P. Westermann review the current technologies and market aspects for short chain fatty acids synthesis by anaerobic fermentation of nonfood biomass, with a focus on a sustainable industrial production. In the first research article, X. Zhu et al. report enzymatic synthesis of precursors of important steroid drugs by biotransformation of cholesterol using *Burkholderia cepacia* strains under different nutritional conditions. In another research article, K. Godlewska et al. report the effects of bioproducts extracted from seaweed on agriculture/horticulture, which comprises increment in plant height and assimilation of microelements and in chlorophyll content. In a third research article, Y. Li et al. report synthesis of pyrimethanil grafted chitosan derivatives with enhanced antifungal activity against plant pathogenic fungi in comparison with chitosan. Finally, Z. Zahan et al. report the effects of anaerobic codigestion of wastes from food manufacturing

and processing companies using municipal wastewater treatment plant's primary sludge and waste activated sludge, and their research suggests that codigestion has great potential in improving the specific biogas production and methane yield with sewage sludge.

In summary and given the importance of raising bioeconomy, the present issue aims to expand our comprehension of different bioprocesses for synthesis of biobased products with economical relevance. In this sense, more than 40 nations are already in the way of establishing a consolidated bioeconomy, which will be a result of some initiatives such as close association of multilateral policy processes and intergovernmental discussions, international collaborations between governments and public and private researchers, international collaboration between researchers to evolve and disseminate the knowledge, and establishment of R&D support programmes [3]. The present issue brings together new potential technologies and methods to lead to an improvement of bioproducts manufacturing worldwide. While meeting those objectives, the work will also provide valuable source of reference for students and researchers.

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Research Article

Anaerobic Codigestion of Municipal Wastewater Treatment Plant Sludge with Food Waste: A Case Study

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The aim of this study was to assess the effects of the codigestion of food manufacturing and processing wastes (FW) with sewage sludge (SS), that is, municipal wastewater treatment plant primary sludge and waste activated sludge. Bench scale mesophilic anaerobic reactors were fed intermittently with varying ratio of SS and FW and operated at a hydraulic retention time of 20 days and organic loading of 2.0 kg TS/m^3·d. The specific biogas production (SBP) increased by 25% to 50% with the addition of 1%–5% FW to SS which is significantly higher than the SBP from SS of 284 ± 9.7 mL_N/g VS added. Although the TS, VS, and tCOD removal slightly increased, the biogas yield and methane content improved significantly and no inhibitory effects were observed as indicated by the stable pH throughout the experiment. Metal screening of the digestate suggested the biosolids meet the guidelines for use as a soil conditioner. Batch biochemical methane potential tests at different ratios of SS : FW were used to determine the optimum ratio using surface model analysis. The results showed that up to 47-48% FW can be codigested with SS. Overall these results confirm that codigestion has great potential in improving the methane yield of SS.

1. Introduction

Sludge production from municipal wastewater treatment plants (MWTPs) is expected to continue to increase with the increasing number of treatment plants being constructed or upgraded due to the growing population connected to the sewage networks of Australia. The disposal of sludge generated at the MWTPs is a problem of increasing importance, representing up to 50% of the current operating costs of a wastewater treatment plant [1]. In Australia, MWTPs produce approximately 360,000 dry tonnes of stabilised sewage sludge to dispose of which costs about $100 M per year [2, 3]. Hence, water authorities operating these plants in Australia have been actively investigating alternative sustainable and economic sludge management pathways [4]. Although different disposal routes are possible, anaerobic digestion (AD) appears to be the most promising sludge management alternative due to its ability to generate bioenergy by the reduction of the sludge volumes to be disposed of [5–8].

Sewage sludge (SS) contains a high percentage of organic matter (60–70% of the dry matter) and nutrients, and typically comprises primary sludge (PS) and waste activated sludge (WAS) [9, 10]. However, since WAS has low biodegradability; the AD of WAS has low efficiency from both processing and economic standpoints [11]. One of the different strategies to enhance the performance of AD is the codigestion of sludge with other organic wastes as it increases biodegradable organic matter and provides a feedstock with an optimum C/N ratio [1, 4–12]. Among the factors that limit the codigestion are the selection and type of new organic wastes and the transportation cost of cosubstrates to the MWTPs [9–13].

Food wastes (FW) from different sources, for example, residential and commercial, are being produced at an increasing rate due to the growing population and rising living standards [8]. FW is available all year round and accounts for a significant proportion of municipal solid waste. In Victoria Australia, FW contributes 35.6% of the total municipal solid

wastes when source separated, usually referred to as organic fraction of munipal solid waste [14]. However, due to its high biodegradability and volatile acids, AD as a single substrate may encounter various potential inhibitors including accumulation of volatile fatty acids' (VFAs) accumulations [15]. Therefore, these FW could be beneficial in anaerobic codigestion for high energy recovery as well as solid waste reduction.

The application of anaerobic codigestion for the treatment of SS has been receiving growing attention for improving the biogas yield, solid destruction, and the production of digestate of a suitable quality to use as a fertilizer [16]. Full-scale applications of anaerobic codigestion of SS with FW can become an environmentally sound renewable energy source by creating opportunities to recover the energy potential from these very low or zero cost FW and obtain the benefit of high organic matter to increase the methane yield. This will result in significantly less biosolids' disposal and a reduction in municipal solid wastes as well as the operating costs of the plants.

Many authors have reported increased biogas yields from the codigestion of SS with different types of food and/or food processing wastes. For example, codigestion of sludge mix with fat, oil and grease (FOG) from a meat processing plant (46% VS added to the feed) increased the methane yield by 60% [17]. Similarly, methane yield was 2.6 times higher when SS was codigested with oil and grease (48% of total VS load) from restaurants [18]. Under mesophilic conditions, the highest methane production rate was observed when FW was mixed in the range of 30–40% VS with SS [16, 19].

An MWTP in Melbourne, Australia, produces about 3600 kg solids/day of which 627 kg is WAS and the remaid is PS. Since, this plant is in the progress of upgrading the existing old anaerobic digestion reactor, the management was interested in assessing the feasibility of the codigestion of sludge with two streams of wastes, namely, grease trap waste collected from food businesses in the area, referred to in this study as FOG, and waste from a food products manufacturing factory at a small ratio. The MWTP interest is to maximise methane yield, enhance solids removal, and maintain or improve biosolids quality.

The aim of this study, therefore, was to assess the effect of the sludge: waste ratio on the biogas yield and the quality of digestate and supernatant nutrient produced under semicontinuous conditions and, to monitor process performance and stability during codigestion experiments.

2. Materials and Methods

2.1. Characteristics of Substrates and Inoculum. The sludge feedstocks used in this study were thickened PS and WAS collected from Melton Recycled Water Treatment Plant, Victoria, Australia. The PS and WAS were mixed at a ratio consistent with their flow rates such that the final mixed SS's total solids (TS) is 4%. The raw PS and WAS were collected several times while running the experiment and each time they were characterised and mixed as described. The characteristics of different SS samples are reported in Table 1. The SS was stored in a sealed plastic container at 4°C.

The FW used were (i) thickened grease trap, referred to in this paper as GT, obtained from a commercial business that collects FOG from restaurants and food businesses in the western area of Melbourne and (ii) wastes from processed food products manufacturing factory denoted as PF. These FW are mostly comprised cooking oil, butter, cheese, meat, bread, meat fat and bones, mayonnaise, salad dressing, and so forth. The food wastes were collected regularly, homogenised using a high speed homogeniser, and then characterised by the parameters shown in Table 1. The TS of the substrates (SS, PF, and GT) was adjusted such that the AD reactors received a feedstock of consistent TS and chemical oxygen demand (COD) concentration throughout the duration of the experiments. The inoculum used in this experiment was collected from the mesophilic anaerobic digester at Melton wastewater treatment plant. The characteristics of the feedstocks (SS, PF, and GT) and the inoculum are shown in Table 1.

2.2. Batch Experiments. Batch tests were performed to determine the biochemical methane potential (BMP) of the individual substrates (SS, PF, and GT) and mixtures of the SS and FW (a mixture of PF and GT at 50 : 50 w/w) at different ratios. The experimental design is shown in Table 2. All the BMP tests were performed in 500 mL glass bottles at 37°C according to the guideline of Angelidaki et al. [20]. Each reactor contained 4000 mg VS with $VS_{substrate} : VS_{inoculum}$ ratio of 0.25. In addition, two reactors received only inoculum as a control. The headspace of the bottles was flushed with nitrogen gas for 2 minutes and the bottles were closed with a rubber Suba-Seal. All batch tests were performed in duplicate. The bottles were kept at 37 ± 1°C in an incubated shaker at a constant rotational speed of 100 rpm. The volume of biogas produced was measured using a water displacement unit and the biogas composition was monitored using gas chromatography. The volume of biogas (or methane) from the control was subtracted from the volume of biogas (or methane) produced in each reactor to obtain the net production of biogas or BMP from the substrates fed into the reactor.

2.3. Semicontinuous Experiments. SS was mixed with the wastes from the processed food products manufacturing and/or FOG, at the designated ratio of sludge to waste (SS : PF, SS : GT). The experiments were performed in 500 mL glass reactors, designed to allow feeding and nitrogen flushing simultaneously, at 37 ± 1°C in an incubated shaker at a constant rotational speed of 100. The reactors received the substrates at a concentration of 4% TS and operated at an organic loading rate of 2.0 kg TS/m^3·d. The experiment comprised duplicate reactors for each condition. The reactors were operated at a sludge retention time of 20 days (equivalent to hydraulic retention time, HRT, in this case) and were fed and wasted once a day. The biogas was collected before feeding the reactors every day. The biogas measurement, feeding, and wasting were done within a 15 min window out of the incubator. The reactors were monitored weekly for biogas quality, and the wastage was analysed every ten days for pH, TS, and VS, total COD (tCOD), and soluble COD (sCOD). The feedstock to the reactors was prepared from different substrates at the ratios shown in Table 2.

Table 1: Characteristics of substrates and inoculum.

Parameters	Unit	SS	PF	GT		Inoculum
				1st sample	2nd sample	
TS	%	3.7 ± 0.1	18.77 ± 0.8	7 ± 0.2	26.1 ± 0.2	1.85 ± 0.2
VS	%	3.13 ± 0.11	18.06 ± 0.7	6.8 ± 0.16	25.55 ± 0.2	1.32 ± 0.12
tCOD	g/L	53.73 ± 8.2	239.1 ± 0.91	405.3 ± 50	475.5 ± 10	12.9 ± 2.8
sCOD	g/L	3.95 ± 0.6	3.42 ± 0.04	2.98 ± 0.9	3.8 ± 0.7	1.4 ± 0.7
Total N	g/L	2.6 ± 0.1	3.55 ± 0.15	3.5 ± 0.2	3.54 ± 0.2	1.86 ± 0.003
Ammonium	g/L	0.11 ± 0.01	0.11 ± 0.003	0.14 ± 0.01	0.26 ± 0.007	0.48 ± 0.007
Total PO_4^{3-}	g/L	1.5 ± 0.05	1.1 ± 0.04	2.56 ± 0.06	2.58 ± 0.1	0.9 ± 0.3
Total VA	g acetic acid/L	0.6 ± 0.01	1.98 ± 0.15	1.9 ± 0.2	2.03 ± 0.2	0.17 ± 0.013
Alkalinity	g $CaCO_3$/L	2.7 ± 0.001	1.42 ± 0.001	1.3 ± 0.001	2.1 ± 0.01	4.1 ± 0.002
pH		6.36 ± 0.09	5.54 ± 0.01	5.0 ± 0.6	6 ± 0.3	7.55 ± 0.13

Table 2: Composition of the feedstocks used in the BMP and semicontinuous tests.

Experiment type	Substrates in feedstock	Substrates	Composition (w/w)	Nomenclature
Batch	Single	SS	100	100% SS
		PF	100	100% PF
		GT	100	100% GT
Batch	Two	SS : PF	99 : 01	1% PF
		SS : PF	98 : 02	2% PF
		SS : PF	90 : 10	10% PF
		SS : PF	75 : 25	25% PF
		SS : PF	50 : 50	50% PF
		SS : GT	99 : 01	1% GT
		SS : GT	98 : 02	2% GT
		SS : GT	90 : 10	10% GT
		SS : GT	75 : 25	25% GT
		SS : GT	50 : 50	50% GT
Batch	Three	SS : PF : GT	95 : 2.5 : 2.5	5% FW[#]
		SS : PF : GT	80 : 10 : 10	20% FW[#]
		SS : PF : GT	50 : 25 : 25	50% FW[#]
		SS : PF : GT	33.3 : 33.3 : 33.3	66.67% FW[#]
Semicontinuous	Single	SS	100	100% SS
Semicontinuous	Two	SS : PF	99 : 1	1% PF
		SS : PF	98 : 2	2% PF
		SS : GT	99 : 1	1% GT
		SS : GT	98 : 2	2% GT
Semicontinuous	Three	SS : PF : GT	95 : 2.5 : 2.5	5% FW[#]

[#] FW = mixture of PF and GT at ratio 50 : 50 (w/w).

2.4. Analytical Methods. TS and VS were measured by gravimetric analysis according to the Standard Methods 2540B and 2540E, respectively [21]. tCOD and sCOD were measured according to HACH method 8000. The total phosphorus (TP), total nitrogen (TN), ammonium, and volatile acids (VAs) were measured by colorimetric techniques using a HACH spectrophotometer (Model DR/4000 U) according to the methods 10127, 10072, 10031, and 8196, respectively. The samples were centrifuged (Eppendorf 5702, Germany) at 4.4 rpm for 15 mins and then filtered through 0.45 μm filter paper (mixed cellulose esters membrane filter, Advantec, Japan) to measure the soluble constituents. The measurement of pH was carried out using a calibrated pH meter (ThermoOrion, Model 550A) and alkalinity was measured by the APHA method 2320B.

The volume of biogas was normalised to standard conditions compromising dry gas, standard temperature, and pressure (0°C and 1 bar) according to the method described by Strömberg et al. [22] and the results are presented as norm-litre (L_N). The headspace was corrected for methane (CH_4) and carbon dioxide (CO_2) to 100% according to VDI 4630 (2006) [23]. The composition of the biogas was analysed according to APHA method 2720C using gas chromatography (Varian 450-GC, Varian Australia Pty Ltd., Netherlands)

equipped with a packed column (GS-CarbonPLOT 113-3132, 1.5 microns, 30 m ∗ 0.320 mm, stainless steel, Agilent Technologies Inc., Australia) and a thermal conductivity detector. The carrier gas used was helium at a flow rate of 28 mL/min. The temperatures of the column, detector, and injector were 70°C, 200°C, and 100°C, respectively. The biogas was collected and manually injected using a 50 mL FORTUNA® Optima glass syringe (Poulten & Graf, Germany). Calibration was done using three points and five levels of CH_4, CO_2, and nitrogen (BOC, Australia). Screening of the metals in the digestate samples was tested for sodium (Na) to cerium (Ce) by a commercial laboratory (ALS Environmental Division: Water Research Group).

2.5. Statistical Analysis. Predictions of the optimum mixture ration for two and three substrates from batch tests were obtained using MATLAB R2013b. Furthermore, a predictive model for optimum FW incorporation was prepared with surface and contour plots. To determine the significance of difference in cumulative methane yields over the digestion period, each set of codigestion feedstock was statistically analysed with 100% SS using one-way analysis of variance (ANOVA) at $\alpha = 0.05$ in MATLAB R2013b.

3. Results and Discussion

3.1. Batch Experiments. Batch experiments were carried out to investigate the optimum ratio of FW for incorporation in SS. The effects of two substrates and three substrates were also investigated at different mixture ratios. The cumulative methane yields and the daily biogas yields during the anaerobic codigestion are shown in Figures 1(a)–1(d) and 1(e)–1(h), respectively. The BMP tests continued for 46 days until little or no biogas production was observed. The results presented are the net biogas and methane yield from the feedstock after subtracting the control yield.

According to Figure 1(a), the BMP of 100% SS was 192 ± 12.3 mL_N CH_4/g VS_{added}, whereas the processed food wastes, 100% PF and 100% GT, had a BMP of 466.2 ± 0.73 and 408.7 ± 6.6 mL_N CH_4/g VS_{added}, respectively, which is 1.42 and 1.12 times higher than 100% SS alone. For 100% SS, the biogas production started after 2 days and reached the first peak at day 8 with a rate of 21.5 mL_N biogas/g VS_{added}·d (Figure 2(e)). The second peak occurred at day 17 with a peak value of 46.1 mL_N biogas/g VS_{added}·d and after 21 days slowly decreased. Both food wastes started biogas production after day one and reached the first peak at day 17 with daily biogas yields of 54.3 and 45.4 mL_N biogas/g VS_{added}·d, respectively, for PF and GT. The second peak values were 56.3 and 25.3 mL_N biogas/g VS_{added}·d for PF and GT, respectively, at days 28 and 36. The technical digestion time, that is, $T_{80–90}$ (the time for 80–90% of the maximum biogas production), was calculated to be between 20 and 27, 31 and 35, and 37 and 40 days for SS, PF, and GT, respectively. The technical digestion time can be used as a HRT for continuous anaerobic digestion for these substrates [24].

The codigestion of SS with PF enhanced the BMP from 199.6 ± 20.6 to 616.8 ± 30.2 mL_N CH_4/g VS_{added} for PF fractions of 1% to 50%, that is, 4% to 287% increase in methane yield compared with 100% SS alone (Figure 1(b)). However, with 1% PF to 10% PF incorporation, a lag phase of 2 days was observed, and the 25–50% PF mixture with SS immediately started biogas production. For 1% PF to 25% PF, a single peak in daily biogas yield was observed at day 17 with peak values of 36.4 ± 0, 43.9 ± 7.1, 67.2 ± 4, and 86.7 ± 2.7 mL_N biogas/g VS_{added}·d, respectively, for 1% PF, 2% PF, 10% PF, and 25% PF (Figure 1(f)). A rising trend was observed in peak value with increasing PF ratio. The production of biogas was decreased after 20 days and almost ceased after 36 days. However, for 50% PF, an inhibition in biogas production was obtained with two peaks. At day 15, the first peak of 42.4 ± 0 mL_N biogas/g VS_{added}·d with easily degradable organic materials was noted and, at day 28, a small second peak of 29.6 ± 8.7 mL_N biogas/g VS_{added}·d with slow degradation were observed. $T_{80–90}$ was calculated between 20 and 26, 21 and 27, 21 and 27, 21 and 27, and 27 and 33 days, respectively, for 1%–50% PF incorporation.

SS mixed with GT enhanced the BMP of SS from 200 ± 2.6 to 561.3 ± 16.9 mL_N CH_4/g VS_{added}, that is, there was a 5% to 260% increase in methane production, by adding up to 50% GT during codigestion (Figure 1(c)). It was observed that increasing the GT fraction in the feedstock from 1% to 50% caused an increase in BMP up to 17 days and it started decreasing until completely ceased at around 36 days (Figure 1(g)). The peak values were 47.3 ± 2.4, 47.7 ± 3.1, 42.3 ± 1.8, 75.4 ± 13.8, and 89.8 ± 22.8 mL_N biogas/g VS_{added}·d, respectively, for 1% GT, 2% GT, 10% GT, 25% GT, and 50% GT. No inhibition was observed with $T_{80–90}$ between 20 and 26, 20.5 and 26, 21 and 28, 21 and 28, and 25 and 32 days, respectively, for 1%–50% GT incorporation.

For three substrates, biogas production improved with up to 50% FW addition (632.8 ± 10.1 mL_N CH_4/g VS_{added}) and decreased for the mixture ratio of 66.7% FW (603.3 ± 6.7 mL_N CH_4/g VS_{added}) (Figure 1(d)). An early peak at day 8 was observed for 5% FW with a peak value of 40.7 ± 7.9 mL_N biogas/g VS_{added}·d. It was, however, observed at day 17 for 20–66.7% FW with peak values of 63.6 ± 0.5, 74.8 ± 6.8 and 73.3 ± 0 mL_N biogas/g VS_{added}·d, respectively (Figure 1(h)). $T_{80–90}$ was calculated between 24 and 35, 25 and 32, 24 and 30, and 26 and 37 days, respectively, for 5%–66.7% FW incorporation.

Therefore, the addition of FW with SS decreased the technical digestion time with a single peak. The VAs usually associated with the GT appear to be below inhibition up to 50%. However, the inhibition effect at 50% PF indicates that there is NH_4 that reached a threshold (2.1 ± 0.1 g/L). Ammonia which is an important indicator of AD, is produced by the hydrolysis of proteins and urea [25, 26] and accumulates in the AD process [27]. FW was incorporated with SS.

The BMP assay can be utilised to calculate the synergic effect of codigestion as additional methane yield over the weighted average of the individual feedstock's methane yield [28]. The weighted experimental methane was calculated from single substrate using the following formulas

$$\begin{aligned}\text{Weighted EMY}_{\text{FW}} = {} & \text{EMY}_{100\%\,\text{SS}} * P_{100\%\,\text{SS}} \\ & + \text{EMY}_{100\%\,\text{PF}} * P_{100\%\,\text{PF}} \\ & + \text{EMY}_{100\%\,\text{GT}} * P_{100\%\,\text{GT}},\end{aligned} \quad (1)$$

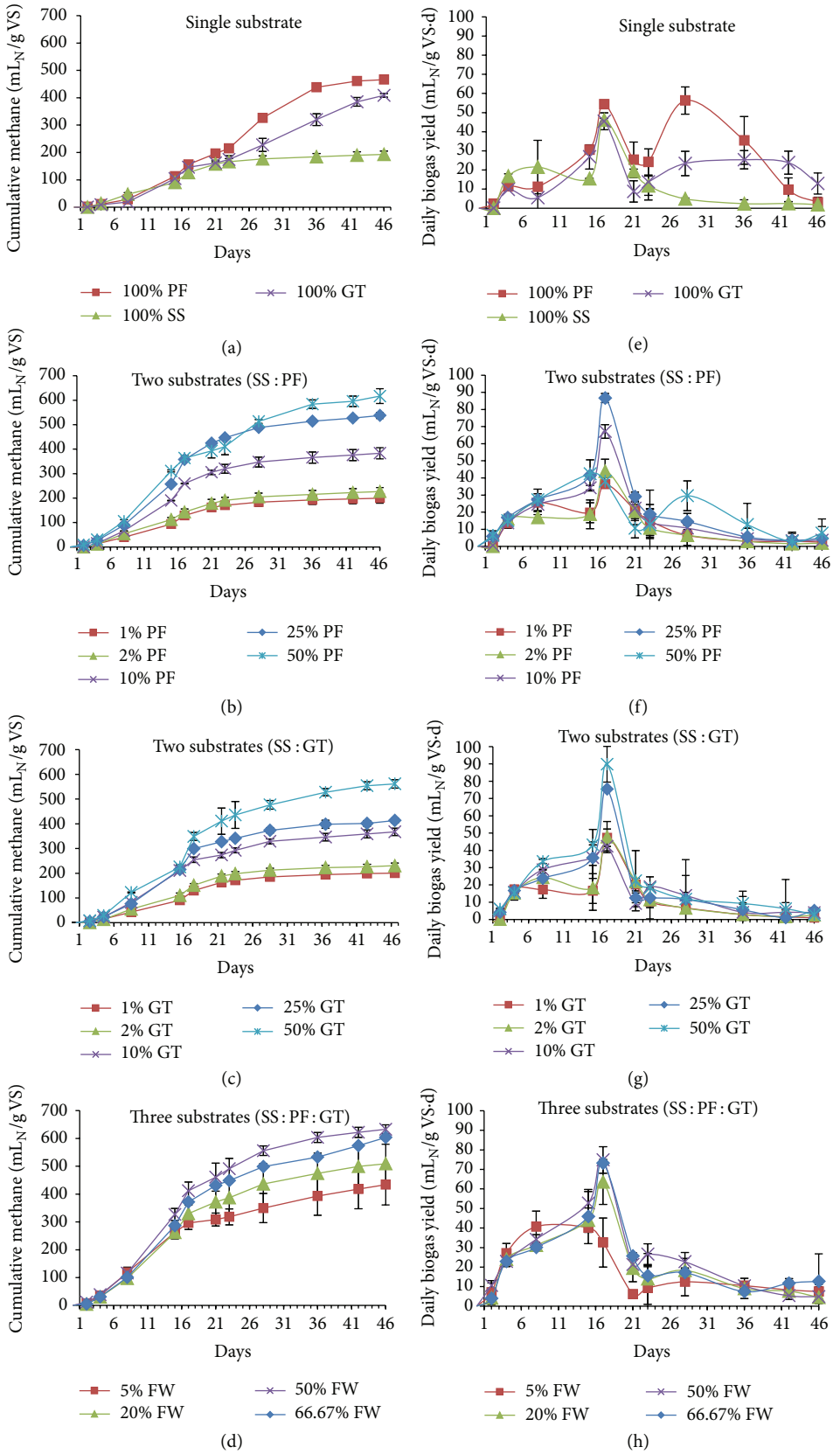

FIGURE 1: Accumulative methane production (a–d) and daily biogas yield (e–f) from batch experiments of single, two, and three substrates.

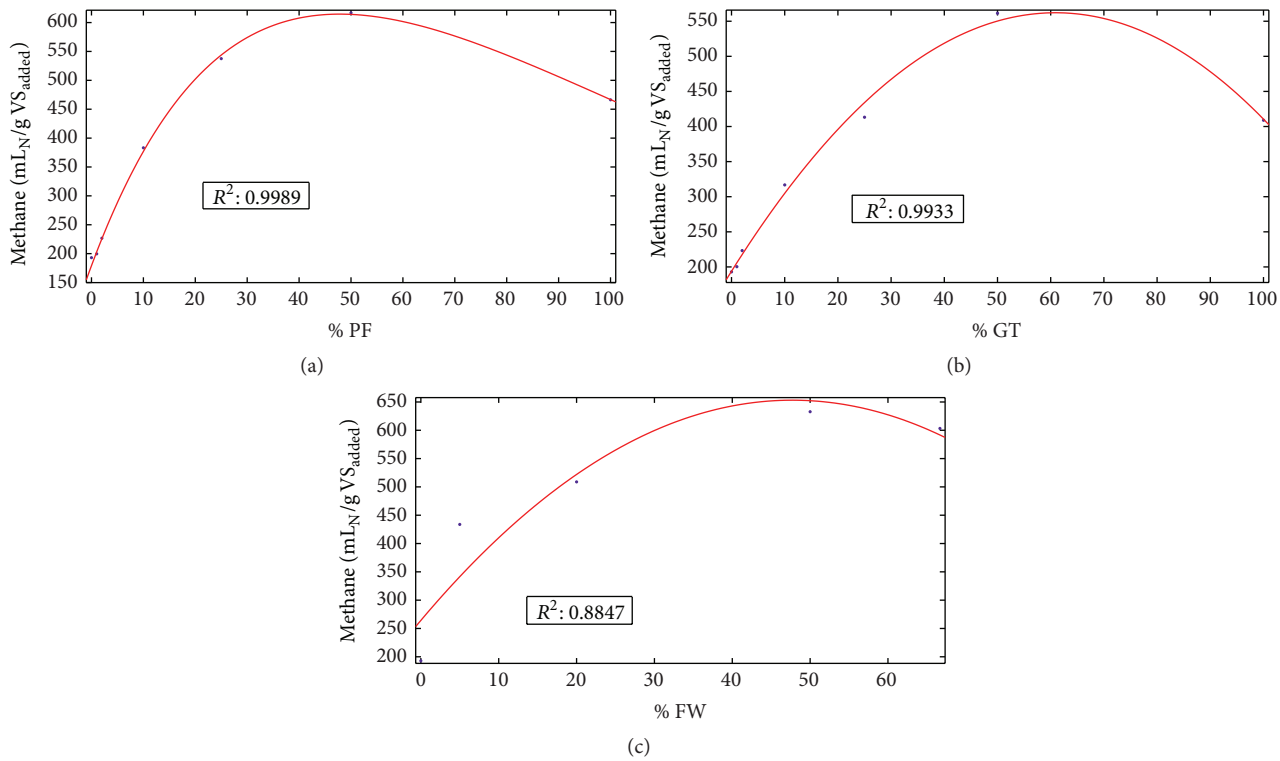

Figure 2: Prediction of optimum SS and FW mix ratio according to the methane yield: (a) %PF, (b) %GT, and (c) %FW.

$$\text{Weighted EMY}_{\text{PF}} = \text{EMY}_{100\%\,\text{SS}} * P_{100\%\,\text{SS}} + \text{EMY}_{100\%\,\text{PF}} * P_{100\%\,\text{PF}}, \quad (2)$$

$$\text{Weighted EMY}_{\text{GT}} = \text{EMY}_{100\%\,\text{SS}} * P_{100\%\,\text{SS}} + \text{EMY}_{100\%\,\text{GT}} * P_{100\%\,\text{GT}}, \quad (3)$$

where weighted EMY_{FW}, EMY_{PF}, and EMY_{GT} represent the weighted average of the experimental methane yield of the substrates FW, PF, and GT, respectively. $P_{100\%\,\text{SS}}$, $P_{100\%\,\text{PF}}$, and $P_{100\%\,\text{GT}}$ refer to the percentage composition and $\text{EMY}_{100\%\,\text{SS}}$, $\text{EMY}_{100\%\,\text{PF}}$, and $\text{EMY}_{100\%\,\text{GT}}$ are the experimental methane yield for substrates SS, PF, and GT, respectively, in the cosubstrates mixture. According to Li et al. (2013) if the difference (EMY − weighted EMY) was higher than the standard deviation of EMY, synergic effect could be observed [29]. The EMYs of the codigestion substrates during the digestion period were analysed statistically with respect to the EMYs of 100% SS. As Table 3 shows, 1-2% PF and GT did not have very significant synergistic effects; however, increasing the amount of food wastes resulted in a very significant ($p < 0.05$) increase in methane yield compared to the digestion of SS alone.

A synergic effect was found in almost all of the cases when food wastes were added to SS representing higher biodegradability. This is possibly due to the adjustment in C/N ratios during codigestion [29] compared to the single substrate. The C/N ratio is a good indicator of the efficiency of AD that can be limited by inadequate amount and diversity of waste from a single resource. For example, high carbon content of a sample can cause rapid acidification and methanogenesis will be inhibited by the low pH. The optimum C/N ratio is waste specific over a range from 9 to 30 [31]. The C/N ratio of SS used in this study was 8.16 which is lower than the C/N ratio of PF and GT (17.64 and 15.5, resp.). Incorporating 50% FW in the feedstock with SS increased the C/N ratio of the reactors up to 12-13. Antagonism (probably due to inhibition) was observed for 50% PF. In case of three substrates, 5% FW showed the highest increase in methane yield. Luostarinen et al. (2009) also reported inhibition with the addition of grease trap sludge to SS of more than 50% [17]. However, these inhibitory effects were only deduced from the pattern of methane production and the synergistic effects and will require further investigations.

To investigate the optimum mixture ratio of FW and SS with respect to methane yield, a trend was predicted using MATLAB (Figures 2(a)–2(c)). The R^2 correlation values were 0.999, 0.993, and 0.885 for %PF, %GT, and %FW incorporation with SS, respectively, indicating a good fit between experimental and predicted values. The results showed that methane yield obtained maximum values of 614.6, 562, and 651.1 $\text{mL}_{\text{N}}\ \text{CH}_4/\text{g VS}_{\text{added}}$ when 47% PF, 61.4% GT, and 48% FW were incorporated with SS improving the C/N ratio of 12.5. Figure 3 shows the 3D model of optimum FW incorporation with SS, where %PF and %GT with SS on the x- and y-axis with methane yield on z-axis. The dark red area represents the maximum methane yield region. FW incorporation up to 48% with the mixture of GT and PF according to the dark red region will produce the maximum volume of biogas. Considering SS as the main substrate, batch

Table 3: Synergistic effect evaluation of codigestion of SS with PF, GT, and FW (mixture of PF : GT).

Substrates ratio[a]	EMY	SD	Weighted EMY	Difference	Increase in EMY (%)	p value	Synergistic effect
1% PF	199.6	20.6	195.7	3.9	2.0	0.9310	Not clear
2% PF	226.6	16.3	198.4	28.2	14.2	0.6106	Not significant
10% PF	383.1	22.9	220.3	162.8	73.9	0.0462	Synergistic
25% PF	537.5	12.3	261.3	276.2	105.7	0.0084	Synergistic
50% PF	616.8	30.2	329.6	287.2	87.2	0.0066	Synergistic
1% GT	200.8	2.6	195.1	5.16	2.7	0.9067	Not clear
2% GT	230.6	10.3	197.3	33.32	16.9	0.5423	Not significant
10% GT	317.3	14.8	214.5	102.8	47.9	0.0467	Synergistic
25% GT	413.2	10.1	246.7	166.3	67.4	0.0259	Synergistic
50% GT	561.3	16.9	300.8	260.5	86.6	0.0081	Synergistic
5% FW	433.7	72.7	205.2	228.5	111.4	0.0176	Synergistic
20% FW	508.9	70.1	241.8	267.1	110.4	0.0110	Synergistic
50% FW	632.8	16.1	315.2	317.6	100.8	0.0038	Synergistic
66.67% FW	603.3	6.7	352.4	250.9	71.2	0.0066	Synergistic

EMY: experimental methane yield (mL/g VS_{added}); SD: standard deviation; and weighted EMY: weighted average of experimental methane yield for cosubstrates.
[a]Percentage of food wastes (PF, GT, and FW) mixed with SS.

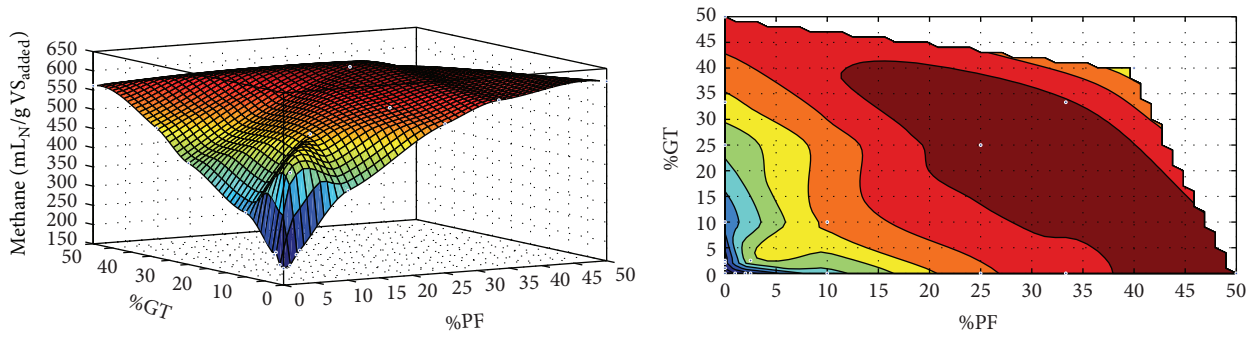

Figure 3: 3D prediction of optimum FW incorporation: (a) surface plot and (b) contour plot.

experiments indicated that mixtures of more than 50% of SS with other substrates can be performed with no risk of inhibition. However, inhibition under continuous operation of a plant also depends on factors such as organic loading rate (OLR), HRT, and reactor configurations. Therefore, a small pilot scale continuousy fed anaerobic digestor should be operated before incorporating the mixture ratio.

3.2. Semicontinuous Experiments. According to the requirement of the plant only 5% or less food waste incorporation was tested for process performances under semicontinuous conditions for six HRT cycles of 20 days each. Figure 4 shows the specific biogas and methane production from the four cycles (20–100 days) reported as mL_N/g VS_{added} fed to the reactor. The average daily methane yield from SS (100% SS) and different mixture ratios of SS with PF and GT (1% to 2%) varied between 212 and 415 mL_N/g VS_{added}. For small amounts of FW incorporation, biogas production was proportional to the percentage of FW and the biogas yield for 5% FW was the highest throughout the experiment duration which is consistent with the BMP assays [11].

For 100% SS, the average SBP was 284±9.7 mL_N/g VS with methane content in the range of 64% and 66%. The average TS, VS, and tCOD removal for 100% SS was 41%, 50%, and 58%, respectively, which was in agreement with COD and VS removal of 35% and 36%, respectively, reported by Silvestre et al. for continuous AD of sludge mix of 70% PS and 30% WAS at an OLR of 1.5 to 1.7 kg VS/m^3·d and HRT of 20 days [9]. A low SBP of 236 ± 6.6 mL_N/g VS was observed during the third HRT cycle (40–60 days) compared to HRT cycle two (20–40 days) when a new batch of feed was prepared with newly collected sample. Low TS, VS, and tCOD removal was also found during the period. This lag phase might be because of the biomass adaptation with the new feed [9]. The pH varied between 6.9 and 7.1 during the whole experiment.

The average SBP of 1% PF and 2% PF was 359 ± 9 and 367 ± 11 mL_N/g VS_{added} which is 25% and 32% higher than the SBP from 100% SS alone. Similarly, 23% and 47% increase in SBP were observed for 1% GT and 2% GT with an average SBP of 355 ± 9 and 367 ± 3 mL_N/g VS_{added}. As FOG has high biodegradability and BMP value (when added below 20% of the influent COD) [13], codigestion with a small

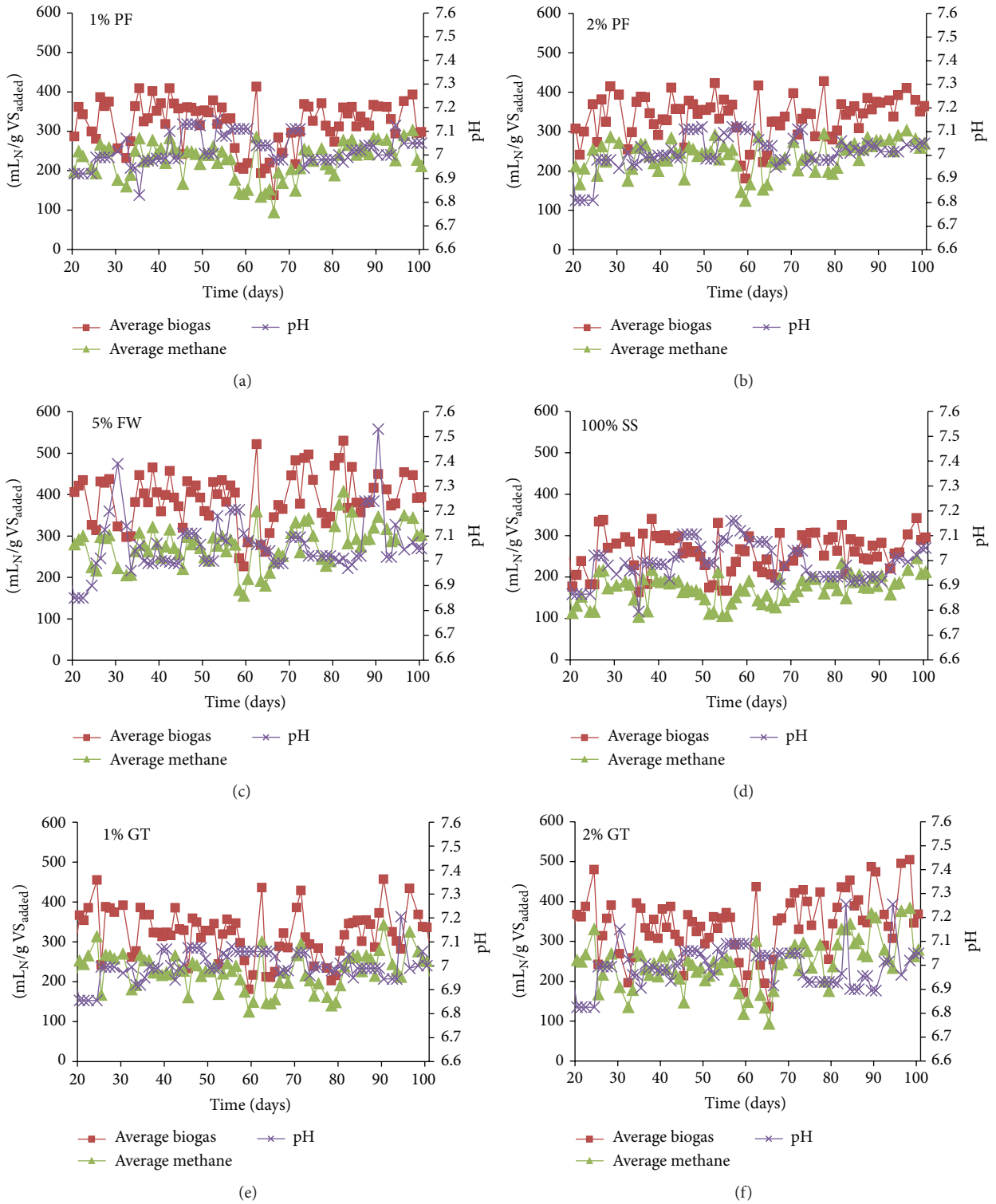

FIGURE 4: Daily biogas production, methane yield, and variation in pH during the codigestion of MWTP sludge with food wastes at different mix ratios: (a) 1% PF, (b) 2% PF, (c) 5% FW, (d) 100% MS, (e) 1% GT, and (f) 2% GT.

proportion of GT produced more biogas than the other food wastes in the same amount. The codigestion of three wastes SS : PF : GT at 95 : 2.5 : 2.5 (5% FW) produced an average SBP of 424 ± 10 mL$_N$/g VS (methane yield 327 mL$_N$/g VS) which is 50% higher than 100% SS (single substrate). These results are in agreement with the results reported by Luostarinen et al. (2009) [17] and Davidsson et al. (2008) [32]. They worked with SS and grease trap sludge (95 : 5 w/w) and reported

Table 4: Biogas production and process performance in terms of TS, VS, and COD removal.

Feedstocks	Parameters	Period I (0–20 d)	Period II (20–40 d)	Period III (40–60 d)	Period IV (60–80 d)	Period V (80–100 d)	Period VI (100–120 d)
1% PF	Avg biogas	256 ± 16	337 ± 14	320 ± 1.5	284 ± 8	339 ± 7	355 ± 9
	CH4%		69 ± 3.2	65 ± 7.81	69 ± 2.8	77 ± 2.8	71 ± 6.7
	TS removal%	43 ± 0.01	43 ± 0.03	40 ± 0.04	40 ± 0.4	46 ± 1	41 ± 4.5
	VS removal%	49 ± 0	51 ± 0.02	49 ± 0.03	45 ± 0.1	57 ± 3.3	50 ± 2.1
	COD removal%	61 ± 0.06	58 ± 0.01	53 ± 0.02	59 ± 0.03	59 ± 0.03	55 ± 2.03
	pH	7.4 ± 0.4	7.0 ± 0.08	7.1 ± 0.08	7.01 ± 0.06	7.02 ± 0.05	7.07 ± 0.03
2% PF	Avg biogas	252 ± 14	335 ± 9	334 ± 2	322 ± 1	364 ± 2	367 ± 3
	CH4%		69 ± 2.4	66 ± 3.4	69 ± 2.4	74 ± 1.4	69 ± 5.4
	TS removal%	43 ± 0.02	45 ± 0.01	41 ± 0.05	45 ± 2.5	46 ± 0.06	45 ± 2.2
	VS removal%	51 ± 0.01	52 ± 0.01	50 ± 0.04	52 ± 0.05	55 ± 0.03	53 ± 1.6
	COD removal%	58 ± 0.1	54 ± 0.01	55 ± 0.06	59 ± 0.03	57 ± 0.03	57 ± 2.05
	pH	7.3 ± 0.4	7 ± 0.04	7.1 ± 0.06	7.01 ± 0.01	7.03 ± 0.02	7.05 ± 0.04
5% FW	Avg biogas	281 ± 1	376 ± 2	386 ± 3	393 ± 2	415 ± 19	424 ± 10
	CH4%		69 ± 5.5	68 ± 7.5	69 ± 7.8	77 ± 4.9	72 ± 5.1
	TS removal%	49 ± 0.01	52 ± 0.02	52 ± 0.08	44 ± 0.06	50 ± 1.0	52 ± 0.07
	VS removal%	56 ± 0.01	60 ± 0.02	60 ± 0.02	55 ± 0.08	54 ± 1.01	59 ± 4.05
	COD removal%	58 ± 0.1	54 ± 0.01	55 ± 0.02	60 ± 0.04	54 ± 0.08	58 ± 1.01
	pH	7.3 ± 0.5	7.09 ± 0.06	7.08 ± 0.08	7.05 ± 0.07	7.1 ± 0.3	7.05 ± 0.04
1% GT	Avg biogas	253 ± 36	285 ± 15	308 ± 7	348 ± 5	345 ± 12	361 ± 1
	CH4%		69 ± 2.51	67 ± 2.3	69 ± 2.12	75 ± 2.8	69 ± 7.1
	TS removal%	44 ± 0.01	45 ± 0.01	45 ± 0.06	43 ± 0.01	44 ± 0.06	45 ± 3.05
	VS removal%	52 ± 0.01	52 ± 0.02	46 ± 0.06	50 ± 1.02	48 ± 0.04	48 ± 5.09
	COD removal%	63 ± 0.04	59 ± 0.01	63 ± 0.04	65 ± 1.06	56 ± 2.3	59 ± 1.06
	pH	7.3 ± 0.5	7 ± 0.06	7.02 ± 0.04	7 ± 0.05	7 ± 0.12	7.08 ± 0.13
2% GT	Avg biogas	284 ± 9	336 ± 5	312 ± 3	329 ± 7	405 ± 4	395 ± 8
	CH4%		68 ± 2	66 ± 5.1	69 ± 4.2	76 ± 4.9	72 ± 4.0
	TS removal%	46 ± 0.02	45 ± 0.01	46 ± 0.04	45 ± 0.01	44 ± 0.02	46 ± 2.04
	VS removal%	54 ± 0.01	54 ± 0.02	53 ± 0.03	53 ± 0.01	57 ± 0.08	53 ± 0.03
	COD removal%	65 ± 0.06	60 ± 0.01	60 ± 0.05	60 ± 0.04	56 ± 0.01	59 ± 0.04
	pH	7.25 ± 0.42	7 ± 0.09	7.03 ± 0.05	7 ± 0.06	7.01 ± 0.2	7.06 ± 0.07
100% SS	Avg biogas	212 ± 1.7	271 ± 5.8	236 ± 6.6	264 ± 3.16	269 ± 3.5	284 ± 9.7
	CH4%		64 ± 4.5	62 ± 1.5	64 ± 7.8	66 ± 2.8	66 ± 9.6
	TS removal%	43 ± 0.02	41 ± 0.03	40 ± 0.06	40 ± 0.01	39 ± 0.03	40 ± 6.08
	VS removal%	50 ± 0.01	54 ± 0.02	46 ± 0.06	50 ± 1.3	51 ± 2.6	53 ± 1.7
	COD removal%	60 ± 0.03	58 ± 0.03	55 ± 0.06	56 ± 0.6	55 ± 0.8	55 ± 1.01
	pH	7 ± 0.12	6.99 ± 0.09	7.04 ± 0.09	6.93 ± 0.02	7.03 ± 0.05	7.05 ± 0.04

methane yield of 374 and 295–308 mL/g VS corresponding to the organic loading of 1.67–2.23 and 2.5 kg VS/m^3·d for HRT of 16 and 13 days, respectively. The addition of food wastes also increased the methane content and the average methane content was 69–72% in this experiment.

The TS removal for 1-2% food wastes (GT, PF) was between 42% and 49% and the corresponding VS removal was found to be between 50% and 56% (Table 4). This is similar to the VS removal reported in previous studies [17, 32]. At the start of the second HRT cycle (20–40 days), the pH was between 6.8 and 6.9 for all the reactors, possibly because of high VA production at the beginning. The pH started increasing after that, indicating the consumption of produced VA due to acidification and inoculum acclimatisation [33]. However, when a new feed was prepared in the fourth HRT cycle (60–80 days), a lag phase was observed with low organic content removal, and low pH as well as low biogas production. However after the lag phase the reactors produced stable biogas production in the last two HRT cycles of the codigestion.

Table 5: Bench scale AD reactors' performance at the end of the experiment.

Parameter	Unit	1% PF	2% PF	5% FW	1% GT	2% GT	100% SS
TS	g/L	21.15 ± 2.43	20.62 ± 2.57	21.13 ± 5.1	20.02 ± 3.46	20.45 ± 0.26	20.34 ± 0.97
VS	g/L	15.67 ± 2.1	17.72 ± 1.61	17.16 ± 1.97	15.33 ± 0.28	14.61 ± 0.26	14.90 ± 2.92
tCOD	g/L	28.025 ± 0.25	26.65 ± 0.07	28.9 ± 0.21	26.075 ± 7.88	28.55 ± 0.21	29.025 ± 5.69
sCOD	g/L	2.05 ± 0.10	1.765 ± 0.06	3.24 ± 0.10	1.755 ± 0.02	2.285 ± 0.04	1.925 ± 0.11
TS removal	%	45 ± 2	47 ± 3	52 ± 5	48 ± 3	48 ± 1	46 ± 1
VS removal	%	52 ± 2.1	53 ± 1.6	55 ± 2	53 ± 0.3	57 ± 0.3	53 ± 3
COD removal	%	57 ± 2.5	59 ± 0.7	59 ± 2.1	60 ± 7.8	59 ± 2.1	55 ± 5.7
TP	g/L	0.36 ± 0.03	0.38 ± 0.04	0.44 ± 0.04	0.4 ± 0.02	0.42 ± 0.01	0.41 ± 0.001
TN	g/L	0.93 ± 0.035	0.895 ± 0.04	0.98 ± 0.035	0.877 ± 0.018	0.965 ± 0.014	0.945 ± 0.035
TKN*	g/L	1.9 ± 0.3	2 ± 0.42	2.4 ± 0.3	2.2 ± 0.2	2.3 ± 0.15	2.2 ± 0.3
NH4-N	g/L	0.62 ± 0.014	0.575 ± 0.035	0.685 ± 0.05	0.7 ± 0.014	0.71 ± 0.014	0.67 ± 0.014
VA	g/L	0.147 ± 0.004	0.148 ± 0.01	0.266 ± 0.07	0.253 ± 0.05	0.315 ± 0.014	0.208 ± 0.005
pH		7 ± 0	7.08 ± 0	7.13 ± 0.02	7.05 ± 0.02	7.09 ± 0.05	7.04 ± 0.02
Alkalinity	g/L	2.498 ± 0.29	2.7 ± 0.06	2.756 ± 0.08	2.711 ± 0.05	2.678 ± 0.03	2.671 ± 0.05

*Analyses were carried out at a commercial laboratory (ALS, Australia).

Methane production was increased significantly from 2% GT after the lag phase, possibly because the methanogens were acclimated to inoculum [4]. However, GT which is mainly lipid-rich material [34] has been found to have wide variation in characteristics (from Table 1, where characteristics' results from two different sample collections are shown).

The daily biogas production was observed to fluctuate, although the feedstocks were prepared by homogenising to constant TS loading throughout the experiments. As the FW had high variations in their characteristics, feeding a very small proportion in the reactors every day (from a batch of prepared feedstock) resulted in variations. Therefore, the average biogas production over each HRT cycle is shown in Table 4 and a rising trend was observed because of the acclimatisation of the inoculum to the feedstock.

The biogas and methane production potential of the food wastes was very high because of its high fat and protein content. Therefore, the incorporation of FW at very small ratios (1–5%) with SS in codigestion significantly improved biogas production from the SS alone. Although the biogas production improved greatly, VS and COD removal was not improved significantly (Table 4). This was likely due to the huge amount of more slowly degradable and/or inert material in the SS (60% degradable) [17]. The biodegradability of FW on the other hand was probably close to 100% due to the dilution with SS which caused the high biogas production.

Although SBP and methane yield depend on the origin of the substrates, composition, and operational conditions (SRT, temperature), the results reported by Silvestre et al. (2011) [9] and Davidsson et al. (2008) [32] showed a methane yield lower than this study when a small percentage of wastes from the dissolved air flotation unit of a wastewater treatment plant and kitchen grease wastes were added to SS. In addition to the biogas yield, different parameters were monitored at the end of each cycle to assess the quality of the supernatant and digestate (Table 5). It was observed that the pH value remained relatively stable at around 7 throughout the operation of the reactors. The alkalinity in all the reactors was around 2.5 to 2.75 g/L which also indicates no accumulation of VAs and the highest VA was observed from 2% GT (0.315 g/L) which is well below the threshold of inhibition (4 g/L) [35]. The VA accumulation might cause the instability of the process and an inhibition of acetotrophic methanogenesis [13]. However, the VAs in the reactors indicate stable process conditions. Luostarinen et al. (2009) observed total VA accumulation of not more than 0.43 g/L with a high ratio of grease trap to sludge in the feedstock (71% of the feed VS) while working with a mixture of PS, WAS, and grease trap sludge [17]. The ammonia-N content in all the reactors was between 0.6 and 0.71 g/L which is below the inhibition range (1.5–2.0 g/L) [4].

The last aspect to consider in anaerobic codigestion is the possibility of producing high quality compost (or fertilizer). In this case, the dewatered digestate characteristics for heavy metal contents need to be considered when assessing the effect of codigestion [7].

In Australia, the concentration of contaminants present in the biosolids and the microbial quality are two important parameters for biosolids' classifications. Contaminant grade (C1 and C2) and treatment grade (T1, T2, and T3) are the classifications of biosolids based on the factors described where C1/T1 are high quality products and can be used without restriction. According to the EPA guideline, biosolids from wastewater treatment plants are categorised as C2/T3 [30]. Integration among the AD and composting is possible where composting can play the role of curing step to overcome the phytotoxicity limit for VA and ammonia [36]. In the AD reactors, no inhibition of VA and ammonia N was observed. The digestate characteristics were adequate for the production of good quality compost by integrating a simple aerobic poststabilisation and dewatering for biological stability.

In Australia, the regulation of heavy metals in fertilizers of organic origin is governed by the Fertilizer Working Group, Department of Agriculture, AU Government (http://www.agriculture.gov.au/). The concerned heavy metals are zinc (Zn), copper (Cu), nickel (Ni), cadmium (Cd),

Table 6: Digestate heavy metals concentration* in mL/g at the end of the experiment (after six HRT cycle of 20 days).

Parameter	1% PF	2% PF	5% FW	1% GT	2% GT	100% SS	Limit** (mg/kg)
Ca	430 ± 4	455 ± 5	480 ± 6	450 ± 5	440 ± 4	450 ± 7	
Mg	82 ± 1	85 ± 0.5	89 ± 0.7	89 ± 0.4	85 ± 0.3	87 ± 0.5	
Ca hardness	1100 ± 2	1100 ± 0.5	1200 ± 1	1100 ± 0	1100 ± 0.5	1100 ± 0	
Mg hardness	340 ± 0	350 ± 0	370 ± 1	360 ± 2	350 ± 5	360 ± 5	
Al	170 ± 0	170 ± 0	180 ± 0	170 ± 0	170 ± 0	200 ± 0	
As	<1	<1	<1	<1	<1	<1	20
Cd	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1
Cr	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	400
Cu	11 ± 1	11 ± 1	12 ± 0	12 ± 1	12 ± 0	14 ± 0	100
Fe	160 ± 10	160 ± 5	170 ± 10	170 ± 10	170 ± 5	170 ± 5	
Pb	<0.5	<0.5	<0.5	<0.5	0.5 ± 0.1	0.6 ± 0.1	300
Hg	<1	<1	<1	<1	<1	<1	1
Ni	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	60
Zn	19 ± 0.5	20 ± 0.5	21 ± 1	20 ± 0.5	21 ± 1	22 ± 1.5	200
Si	170 ± 5	180 ± 5	170 ± 5	170 ± 5	180 ± 5	210 ± 10	
Si-SiO2	350 ± 10	380 ± 5	370 ± 5	370 ± 5	380 ± 5	440 ± 10	
S	160 ± 5	170 ± 0	180 ± 5	170 ± 5	170 ± 5	200 ± 5	
S-SO4	480 ± 10	520 ± 5	540 ± 5	520 ± 5	520 ± 5	600 ± 10	

*Heavy metal screening of the digestate samples was carried out by a commercial laboratory (ALS Environmental Division: Water Research Group).
**Contaminant upper limits for biosolids as grade C1 [30].

lead (Pb), chromium (Cr), and mercury (Hg) and their allowable limits are shown in Table 6.

These heavy metals may be present in concentrations above the legal limits which can potentially harm environment and affect crop quality, crop yield, and soil fertility. Heavy metal concentration may increase during AD due to the microbial mineralization and loss of volatile solids [37]. Most national regulations prohibit the use of organic fertilizers, for example, digestate, if the concentrations of one or more heavy metals are higher than the threshold concentrations. There is also evidence suggesting that AD increases the complexation of heavy metals with organic ligands and hence lowers the mobility of heavy metals in the digestate [38, 39]. However, the metal contents found in these experiments were less than the allowable limit used in Australia for high quality amendments. Table 6 shows the concentration of heavy metal in the digestate collected reactors after six HRT cycles (at the end of the experiment).

4. Conclusions

FW is a suitable cosubstrate for the anaerobic codigestion of SS. The addition of 5% FW to the SS increased the SBP by up to 50% during semicontinuous experiments. Although the TS, VS, and tCOD removal slightly increased with codigestion, the methane content of the bioga improved significantly. The reactors showed stable pH and performance with no inhibitory effect. Based on the results from batch assays and the use of surface modelling, FW can be added at ratios up to 47%-48% (v/v) without inhibition to the AD process. Overall these results reveal the high potential of codigestion FW with SS to enhance bioga yield and quality.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Microbial Production of Short Chain Fatty Acids from Lignocellulosic Biomass: Current Processes and Market

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Biological production of organic acids from conversion of biomass derivatives has received increased attention among scientists and engineers and in business because of the attractive properties such as renewability, sustainability, degradability, and versatility. The aim of the present review is to summarize recent research and development of short chain fatty acids production by anaerobic fermentation of nonfood biomass and to evaluate the status and outlook for a sustainable industrial production of such biochemicals. Volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid have many industrial applications and are currently of global economic interest. The focus is mainly on the utilization of pretreated lignocellulosic plant biomass as substrate (the carbohydrate route) and development of the bacteria and processes that lead to a high and economically feasible production of VFA. The current and developing market for VFA is analyzed focusing on production, prices, and forecasts along with a presentation of the biotechnology companies operating in the market for sustainable biochemicals. Finally, perspectives on taking sustainable product of biochemicals from promise to market introduction are reviewed.

1. Introduction

In 1996, the report "Technology vision 2020" [1] was published by the US chemical industry. The work was promoted by the White House Office of Science and Technology Policy and focused on future needs in research and development (R&D). But the report only pinpointed market issues as the important challenges for the chemical industry towards 2020. Three years later, a second report was published [2] with a completely different content and perspective for the chemical industry: New Biocatalysts, Essential Tools for a Sustainable 21st Century Chemical Industry. The report promoted government initiatives and included the extensive studies that had been carried out by the Pacific Northwest National Laboratory (PNNL), in collaboration with the National Renewable Energy Laboratory (NREL), and by the Office of Biomass Program. In 2004, their first report was published, entitled Top Value Added Chemicals From Biomass [3]. The ultimate task was "...to identify the top ten opportunities for the production of value-added chemicals from biomass that would economically and technically support the production of fuels and power in an integrated bio-refinery and identify the common challenges and barriers of associated production technologies."

Initially, the authors developed a catalogue with a list of more than three hundred putative building block molecules, all having the potential for biocatalytic production from biomass. The list was then narrowed down to end up with almost fifty potential building block candidates. Among those were VFAs such as acetic acid and propionic acid. Acetic acid was chosen as commodity chemical and as a reagent adding functionality to hydrocarbons by supplementing with two carbon units. Propionic acid was selected as a reagent and a building block compound.

Ultimately, the authors identified and listed fifteen chemicals that could be produced from carbohydrates and suggested the compounds as targets for intensified scientific research. The choice of targets was based upon factors such as established conversion processes, the ability of a compound to serve as a platform for the production of derivative compounds, industrial viability, and economic aspects such as market size [4]. Although VFAs were not included among the

ultimate top fifteen compounds, the 2004 report resulted in an increased research in sustainable organic acid production [5].

VFAs are widely used building block chemicals, which are employed in the manufacturing of a wide range of chemicals, pharmaceuticals, and materials, or they are used as free acids for, for example, feed conservation in the agricultural industry.

Acetic acid and derivatives are applied in a range of industries such as the electronic industry, polymer industry, chemical industry, and the food industry. The acid has many uses including the following: as an etching agent [6], as a component in detergents used for manufacturing of microelectronics; in the production of lignin-containing polyurethane [7], as a component in the manufacturing of hydrophobic and lipophobic papers in the polymer industry [8], in polyethylene production, and as an important preservation ingredient in the food industry. The acid is the principal compound in vinegar [9] and is used in numerous industrial and household food products and preparations [10].

Propionic acid and its derivatives are used either directly or as building block chemicals in a range of industries and in agriculture. Propionic acid is used without modification as a preservative in the food industry. The acid and its salts, such as sodium and calcium propionate, are used in agriculture for animal feed and grain preservation [11, 12]. A recent study on feed preservation demonstrated that treatment with a combination of acetic acid and propionic acid conserved well and prolonged storage in comparison to a nontreated control [13]. Moreover, propionic acid is used directly or as a modified compound in the manufacture of herbicides [14], as building block in pharmaceuticals [15] and in cellulose acetate propionate (CAP) plastics in the polymer industry [16].

Swedish company Perstorp Speciality Chemicals AB is marketing a product consisting of propionic acid and propionic acid glycerol esters. The mixture is an animal feed preservative that inhibits growth of molds and yeasts at a dosage of around 0.5–2.7% in stored grains [17].

Butyric acid and its derivatives have broad applications within the food industry, perfume and fragrance industries, the polymer industry, and the pharmaceutical industry.

Food and beverage industries use butyric acid directly to add or enhance a butter-like taste in food and beverages. Esters of butyric acid are generally aromatic and due to their fruity fragrance, they are used as additives for increasing fruit fragrance and as aromatic compounds in the production of perfumes [18]. Butyric acid is also used to synthesize butyryl polymers such as cellulose acetate butyrate (CAB) in the polymer industry [19]. In human health applications, butyric acid is a component of prodrugs with demonstrated anticancer effects [20]. Furthermore, derivatives of butyric acid from endogenous bacteria are known to promote colon health, and the acid presumably also has therapeutic clinical effects [21]. Currently, most VFAs used in industry are produced by petrocatalysis from refined heavy oil and natural gas. Naphtha and syngas are examples of such refined products, which might be further refined and processed by organic catalysis and synthesis to VFAs [22] that in turn serve as platforms for further organic synthesis based upon reaction with the terminal carboxylic group of the acids. Petrocatalysis involves high pressure and temperature process conditions requiring high energy inputs [23] and about 4% of global oil consumption is currently related to the production of chemicals and plastics [24]. Although this is a relatively low figure, VFA production from oil creates hazardous wastes such as heavy metals and organic solvents in addition to the emission of greenhouse gasses.

Acetic acid is produced mainly from mineral oil and natural gas either through methanol carbonylation or acetaldehyde oxidation [22].

The primary route of propionic acid synthesis employs the Oxo-synthesis process by hydrocarboxylation of ethylene in the presence of a nickel carbonyl catalyst or a rhodium catalyst but liquid-phase oxidation of propionaldehyde also yields propionic acid [22].

Butyric acid can be prepared chemically by oxidation of butyraldehyde obtained from propylene by Oxo-synthesis similar to propionic acid synthesis [25].

However, oil-derived chemicals can be produced from biomass instead [26], because, for example, lignocellulosic biomass has a chemical composition similar to fossil feedstocks and pretreated lignocellulosic biomass offers chemical compounds comprising different functional groups that facilitate chemical processing [27].

VFA can also be synthesized by microbial fermentation that requires three distinct process units: (1) biomass conversion, (2) fermentation, and (3) the recovery of the products from the fermentation broth [28, 29]. Even though petrocatalysis has been used for many years, there might be reasons to shift to biological processes instead. However, the sustainability of microbial conversion is still of major concern.

2. Sustainability of Biomass-to-Chemicals Process

If biomass-to-chemicals processes employ renewable feedstocks in integrated unit operations with recycling and exhaustive use of raw materials and energy, then they meet a rough definition of a biorefinery.

To become specific, biomass feedstocks must have important potential advantages over fossils. For example, carbon emitted to the atmosphere from conversion of renewable biological materials has a net zero carbon impact on the atmosphere's chemical composition [30, 31]. In other words the carbon is part of a closed loop whereby plant growth recaptures the carbon that is emitted during biomass use and its conversion. In addition to this, plant biomass is usually a domestic resource, which can be obtained at little cost [32]. Indeed, one requirement is that the biomass is of second-generation origin and has been grown and harvested without upsetting food supplies and supplies of feed and fiber [33]. Moreover, biomass conversion to organic chemicals such as VFA requires several chemical reactions in succession, which in turn require energy. Therefore, carbon neutrality can only be achieved if the energy,

that is used to power the processes, is from renewable sources.

Although there are exceptions to the notion that biomass use is carbon neutral, especially within the context of biofuels and bioethanol [34], the notion is rarely challenged [35]. But even biomass from dedicated crops might not be carbon neutral. On the contrary, agriculture can potentially increase atmospheric CO_2 because emissions are highly dependent on where and how the biomass is grown and harvested [31, 35, 36].

A thorough discussion of carbon footprint and putative carbon neutrality in the production of biochemicals is beyond the frame of this review. What should be mentioned though is the difference to an overall carbon footprint between direct combustion of lignocellulosic biomass and the carbon capture by incorporation of carbon into chemicals. To some extent, the latter alternative makes up a contemporary carbon sink.

Interest in the biomass-to-chemicals value chain has increased sharply during the recent ten years within industrial companies. Such interest has accelerated R&D into development of sustainable biomass conversion-to-chemicals processes [37] and there are specific factors on the production side in favor of sustainably produced chemicals to substitute for petrochemical counterparts. When chemicals are produced from biomass, the biorefinery saves energy and mitigates CO_2 emissions [38] and it has been put forward that industrial biorefineries, as opposed to oil refineries, often show higher reaction rates, increased conversion efficiencies, improved product purities, and reduced chemical waste generation [39]. On the demand side, consumers wish for "natural" and "green" preservatives, fragrances, and materials [40]. In some markets, consumers prefer food additives or pharmaceutical products containing ingredients of natural origin. They are considered "healthier" and customers are often ready to pay more for such natural products. Moreover, biomass-derived chemicals are considered safer for human health than oil-derived products [41, 42].

A frequent argument put forward in favor of transition away from petrochemistry toward biochemistry is the rising oil prices and the finite nature of oil reserves. According to the International Energy Outlook 2013 [43], global energy consumption will grow by 56 percent between 2010 and 2040 and fossil fuels are predicted to continue to supply almost 80 percent of world's energy demand towards 2040. As a consequence, global energy-related carbon dioxide emissions are projected to increase by 46% in the same period of time given the current policies and regulations.

Currently, about 95% of all manufactured chemicals originate from fossil resources and only around 5% from renewable resources [44] that in principle are unlimited because they can be replaced over time, and only a fraction of this is from biomass conversion in microbial processes.

Also public and political concern about the volatile global oil market has been raised to advocate for the need to use renewable resources instead. It is a strong argument as far as transportation fuels are concerned, but it is less convincing for the manufacture of biochemicals because less than 5% of the global oil consumption is required to synthesize chemicals. In the US it is around 3% [45].

3. Microbial VFA Processes from Biomass

Biological catalysis from renewable feedstocks may be an attractive alternative to petrochemical multistep reactions that are employed in traditional VFA synthesis. The total energy input is generally reduced in biocatalysis compared to petrocatalysis because biocatalysis is occurring at low temperature and low pressure. Moreover, use of heavy metals is abandoned, the use of organic solvents and strong acids and bases is reduced, and fewer by-products are produced by biocatalysis because microbial enzymes are highly specific [46–48]. But downstream processing such as recovery of acids from the fermentation broth is a challenge both technically [49, 50] and economically [51].

Biomass conversion in a sustainable and biological production of chemicals can proceed by either of two routes in a biorefinery (Figure 1). Feedstock comprising, for example, plant biomass may be processed either via carbohydrate extraction, which constitutes the sugar platform, or via gasification of the biomass, which constitutes the syngas platform. In the first case, the sugar platform is basis for fermentation of C5 and C6 sugars and the produced metabolites such as VFA may be readily used or subjected to subsequent upgrading by chemical catalysis [52]. In the second case, the syngas is converted in gas fermentation predominantly with fuels as principal products [53, 54]. Biomass conversion to intermediate VFAs by anaerobic digestion, which in turn are converted into biogas, is also feasible [55] but is not treated in this review.

Anaerobic VFA production is a result of microbial fermentation where the enzymes convert various carbon substrates to energy (ATP), reducing agents, and intermediates used in anabolic processes and a number of metabolites such as acetic, propionic, and butyric acids. Acetic acid is produced anaerobically through the glycolytic pathway and via pyruvate intermediates as a coproduct by a number of organisms that produce, for example, propionic and butyric acids as their major products. *Propionibacterium* and *Clostridium* are examples of genera capable of these transformations. A conventional fermentation route to acetic acid is via ethanol using, for example, *Acetobacter aceti* [56]. Direct aerobic acetic acid production is conducted by using *Escherichia coli* as biocatalyst in renewable sugar fermentation [57], and *Saccharomyces cerevisiae* has also been used for this purpose [5]. Propionic acid biosynthesis by *Propionibacterium* species takes place via a glycolytic and then a dicarboxylic production pathway through pyruvate and succinate intermediates [58]. Butyric acid is the end product from metabolism of sugars via glycolysis and then through a dicarboxylic production pathway via pyruvate [59].

The split pathways in both propionic and butyric acid fermentations also yield acetic acid (and ATP) that drains the carbon pool, which in turn affects both the final titers and the final recovery of propionic and butyric acids.

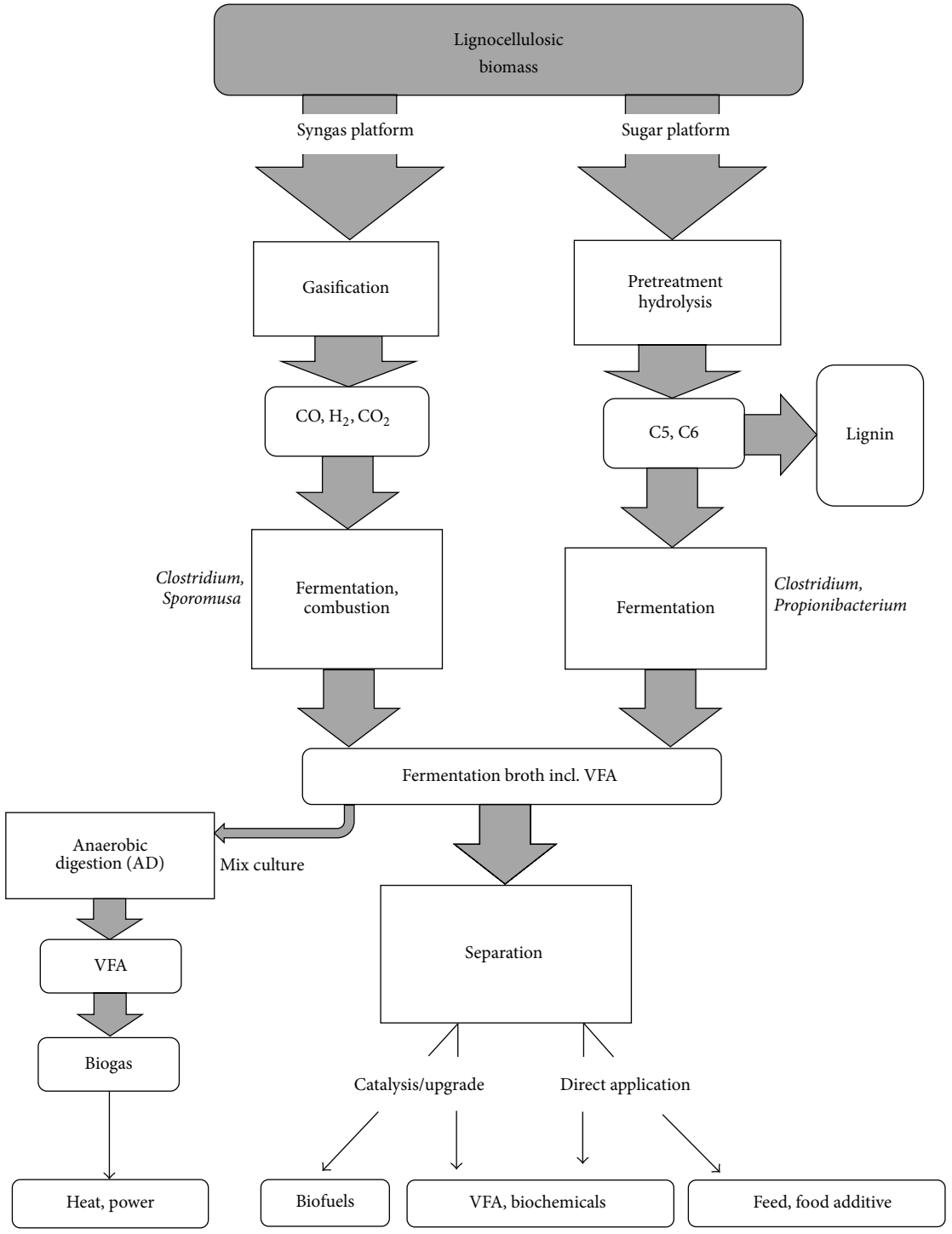

Figure 1: Routes for biomass conversion via one of two platform processes. The sugar platform via pretreatment to microbial fermentation and the syngas platform via gasification to microbial fermentation or combustion. Process feedstock is lignocellulosic biomass and fermentation products are VFA, other biochemicals, and biofuels.

The current state of biomass conversion to VFA by microbial processes is still limited. Anaerobic fermentation of plant biomass-derived carbohydrates for direct production of acetic acid is currently not a significant R&D topic, probably because high-value acetic acid is cheaply derived from aerobic conversion of alcohol-containing solutions produced from sugar-containing juice fermentation by acetic acid producers.

The most important organisms used for this process are aerobic bacteria belonging to genera such as *Acetobacter* and *Gluconacetobacter* that produce up to 150 g/L [60]. The food-grade solution derived from this process is vinegar,

which is a high-value commodity. Extensive R&D is conducted to improve biological acetic acid production using, for example, acid and thermotolerant strains in aerobic submerged fermentations [61] at different oxygen concentrations [62], with adapted or transformed strains expressing heterologous genes conferring acid tolerance [63], or with genes that promote production capabilities in organisms such as *A. polyoxogenes* that produces around 100 g/L with a rate of 4 g/L/h [64].

Conversion of biomass to acetic acid without the process of microbial fermentation has been investigated [65] and it is well established that biomass degradation using harsh methods leads to formation of significant amounts of acetic acid and other compounds by partial degradation of the plant cell constituents [66]. It is, however, feasible to produce acetic acid from plant biomass by employing mild solvents such as super- and subcritical water instead [67]. For example, increased yields and purity of acetic acid was obtained in an oxidation process conducted at low pressure and without an acid catalyst [68]. The energy demand of such a process is made up of the requirements for oxygen and heating. Lastly, efficient utilization of monoculture microbial conversion of plant biomass by some kind of coproduction [69] of acetic acid and other commercially interesting metabolites has not been reported either through the use of acetic acid bacteria or by using other anaerobic bacteria. However, Brazil based company Braskem has taken out a patent for anaerobic coproduction of acetic acid and isoprene by a genetically modified microorganism in a sustainable process [70].

Propionic acid is the principal fermentation product of bacteria belonging to the *Propionibacterium* genus and propionic acid production from cheese whey substrate, or other lactose effluents, has been studied since 1923. The conclusions from these studies were very often that the slowness of the process was unacceptable for industrial use except in, for example, cheese production [71]. However, propionic acid production from carbohydrate substrates other than lactose is feasible.

Propionic acid production by *Propionibacterium freudenreichii* cells from sugar cane molasses and waste cells was studied in plant fibrous-bed bioreactors (PFB). With nontreated molasses as carbon source, 12.69 g/L of propionic acid was achieved in 120 hours in stirred fermentation, whereas fed-batch fermentation of hydrolyzed molasses in PFB yielded 79.81 g/L of propionic acid within 302 hours fermentation at a rate of 0.26 g/L/h [72] with recycled cells as a nitrogen source.

In fed-batch fermentation with wild-type *P. acidipropionici* cells and corncob molasses containing high concentration of xylose as substrate, the metabolism resulted in a final 71.8 g/L of propionate and a productivity of 0.28 g/L/h [73].

Glycerol is a residual product from biodiesel production and is commonly used in anaerobic fermentations. In two studies [74, 75], improved propionic acid production was obtained by using a metabolically engineered *Propionibacterium jensenii* strain transformed with a plasmid expressing heterologous glycerol dehydrogenase, which is a required enzyme for conversion of glycerol. In a potential-shifted, fed-batch fermentation, a propionic acid concentration of 39.56 g/L was achieved with a productivity of 0.183 g/L/h.

Low-cost feedstocks were used in a study where propionic acid and vitamin B12 were coproduced from hydrolyzed corn stalks, corn steep liquor, and glycerol [76]. *P. freudenreichii* subsp. *shermanii* cells were exploited to ferment the mixture, which resulted in 42.7 g/L of propionic acid and a productivity of 0.36 g/L/h. Moreover, *in situ* removal of propionic acid by use of an ion exchange resin during fermentation kept the propionic acid concentration at 10 g/L and resulted in propionic acid concentrations of 91.6 g/L after 258 hours of fermentation yielding 0.71 g/g and a productivity of 0.35 g/L/h.

Propionic acid production was increased by an acid adapted *P. acidipropionici* mutant strain when limiting metabolites were identified and supplemented [77]. These were lactate, fumarate, and succinate metabolites that are known to influence propionic acid synthesis. In a fed-batch fermentation using glycerol as substrate, propionic acid concentrations reached 35 g/L after around 150 hours of fermentation.

The mesophilic *Clostridium tyrobutyricum* has been the preferred organism for R&D in butyric acid production for many years. In a study from 2009 [78], pretreated molasses were used in fed-batch fermentations with adapted and immobilized *C. tyrobutyricum* cells. The cells produced 55.2 g/L of butyric acid and utilized all three available sugars in the molasses (glucose, fructose, and sucrose). The fermentation yield was 0.46 g/g and the productivity was 3.22 g/L/h. Butyric acid production in fermentations with pretreated sugarcane bagasse hydrolysate has also been reported [79]. They constructed a genetically modified strain with an inactivated phosphor transacetylase gene that grew in diluted bagasse hydrolysate. When cells were immobilized in the reactor and the hydrolysate was fed during the fermentation, the cells produced 21 g/L of butyric acid with an average yield of 0.48 g/g and a productivity of 0.51 g/L/h.

Wild-type *C. tyrobutyricum* cells fermented a combination of sweet sorghum stalks and beet molasses in 1 L fed batch and with the cells in suspension [80]. The fermentation resulted in a final butyric acid concentration of 58.8 g/L with a productivity of 1.9 g/L/h and a yield of 0.52 g/g. In a continuous fermentation with *in situ* removal of produced acids by membrane separation [81], a substrate adapted strain was shown to produce butyric acid from a highly concentrated wheat straw hydrolysate as carbon source and urea as nitrogen source. The productivity, yield, and selectivity were 1.30 g/L/h, 0.45 g/g carbohydrates, and 0.88 g/g acids, respectively.

Other scientific approaches, using other *Clostridium* species and other feedstocks than carbohydrates, have been reported in the study of sustainable butyric acid production. For example, a *C. ljungdahlii* strain was transformed to produce butyric acid from carbon dioxide by introducing genes encoding essential enzymes in the butyric acid pathway and by knockout of genes in butyric acid competing pathways such as acetate and ethanol pathways [82]. Up to 70% of the carbon and electron flow in the transformed strain was

Table 1: Microbial VFA production from lignocellulosic biomass.

Classification	VFA	Biomass	Conditions	Productivity	Reference
C. cellulolyticum	A.A.	Rice straw	Sugar platform	*Y*: 0.23 g/g	[83]
C. tyrobutyricum	B.A.	Rice straw	Sugar platform detoxification	*T*: 8.7 g/L	[84]
C. tyrobutyricum	B.A.	Wheat straw Switch grass	Sugar platform pH control	*Y*: 0.44 g/g *Y*: 0.42 g/g	[85]
C. tyrobutyricum	B.A.	Wheat straw	Sugar platform acid removal urea supplement	*R*: 1.30 g/L/h *Y*: 0.45 g/g *S*: 0.88 g/g	[81, 86]
C. tyrobutyricum	B.A.	Corn fibre	Steep liquor suppl.	*R*: 2.91 g/L/h *Y*: 0.47 g/g	[87]
C. tyrobutyricum	B.A.	Rice straw	Sugar platform detoxification	*T*: 8.1 g/L	[88]
C. thermobutyricum	B.A.	Sorghum bagasse	Sugar platform 50°C	*T*: 17.6 g/L *Y*: 0.44 g/g	[89]
Mixed culture	B.A.	Rice straw	Sugar platform pH buffered	*Y*: 0.38 g/g *S*: 0.70 g/g	[90]
T. fusca	B.A.	Corn stover	Cellulolytic activity aerobic fermentation 55°C	*T*: 2.37 g/L *Y*: 0.52 g/g	[91]

A.A. denotes acetic acid; B.A. denotes butyric acid. *R*: denotes rate; *S*: denotes selectivity; *T*: denotes titer; *Y*: denotes yield.

diverted to production of butyric acid with either H_2 or CO as electron donors.

VFA production from microbial conversion of lignocellulosic feedstock via the sugar platform is summarized in Table 1. To our knowledge, there are no publicly available reports on microbial VFA production from lignocellulose of propionic acid or VFA production via the alternative syngas platform.

4. The Market for Biochemicals in the Bioeconomy

While the pace of innovation of alternative energy technologies has increased markedly during the recent years [92] along with the transition of our energy supply towards a low carbon market [93], we still use hydrocarbons and fossil resource-based chemicals and will probably continue to do so for many years. Industrial biotechnology and related industries will, therefore, become cornerstones in a future bioeconomy in a way with a lower carbon footprint *per capita*.

Industrial biotechnology already exploits the versatility of microbial biosynthesis for the production of many metabolites. The OECD predicts that investments and economic outputs of all types of applied biotechnologies will expand over the coming decades [94]. The main causes for the development of industrial biotechnology in the past were scientific breakthroughs and technological developments, as well as environmental constraints and changes in consumer behavior and demands. For example, the advent, establishment, and growth of modern recombinant DNA technologies have enabled new routes to commercially interesting products via engineered biocatalysts [95–98]. The main causes for driving industrial biotechnology into a future bioeconomy will be somewhat the same as the previous drivers of change, but two additional factors will catalyze the progress and increase the pace. These are the expected growth in the global population [99] that puts constraints on finite natural resources and global climate change.

To address climate change, there is a need to keep a score of the global carbon balance. This will require reducing and replacing the use of fossil resources and over time moving to sustainable raw materials based on residual feedstocks [33, 100], many of which are well suited to biotechnological processing methods. This will demand a development of both green and clean biotechnological processes focusing on efficient conversion of raw materials requiring little input energy and producing a minimum of final waste [98, 101, 102].

The bioeconomy could also create beneficial opportunities for cooperation between sectors that so far have been separated by promoting sustainable development in rural regions having plentiful biomass resources and establishing new linkages between forestry, agricultural, industrial sectors, and universities [103–106] potentially leading to new ways of manufacturing and whole new products [107, 108].

Many stakeholders share interests in obtaining thorough market information concerning chemicals. Such stakeholders include VFA manufacturers, raw material suppliers, end-users of feed, grain and food preservatives, herbicide manufacturers, manufacturers of derivatives and bioplastics, and manufacturing technology providers. But also potential investors in industrial biotechnology for biochemicals require credible market analyses and statistics about consumer demands, market locations, prices, and forecasts. Market data about biochemicals such as VFA are, however, not readily available for the academic world but only from commercial suppliers. Service companies offering market research reports, market analysis, and market forecasts

Table 2: Market information sources.

Source	References	Location
The Essential Chemical Industry	http://www.essentialchemicalindustry.org/	York, U.K.
MarketsandMarkets	http://www.marketsandmarkets.com/	Dallas, US
Cefic	http://www.cefic.org/	Brussels, Belgium
Reed Business Information Limited. Reed Elsevier	http://www.icis.com/	London, UK Amsterdam, NL
TD The Market Publishers, Ltd.	https://marketpublishers.com/	Limassol, Cyprus
IHS, Inc. [109]	https://www.ihs.com/ http://www.chemweek.com/	Douglas County, US
Biofuels Digest	http://www.biofuelsdigest.com/	
Green Chemicals Blog	http://greenchemicalsblog.com/	NYC and London
Biotechnology Industry Organisation	https://www.bio.org/	Washington DC, US
Focus on Catalysts	http://www.journals.elsevier.com/focus-on-catalysts	
Biomass Magazine	http://www.biomassmagazine.com/	Grand Forks, US
iBIB2014/15	http://nova-institut.de/	Hürth, Germany

deliver updated reports on demand against a payment of around USD 5000 for access to a 100–300 pages report. In this review we have collected insights and facts about the market for VFA from open sources primarily, but also from commercial suppliers offering limited information free of charge. In Table 2 a number of useful sources available free of charge are presented.

5. The Market for Biochemicals

In economic terms such as turnover and the number of employees, the global market for chemicals is significant. According to American Chemistry Council, the global chemical production volume rose by around 10% from 2012 to 2016 [110].

The majority of the chemicals produced are carbon-containing compounds that are supplied from refining of fossil feedstocks. According to a forecast by The European Chemical Industry Council [111], European chemicals industry will remain oil-based over the next decades [111] but, as Cefic points out, there is untapped potential for increased use of biobased feedstocks, not only for the production of specialty chemicals but also of high-volume building block chemicals such as the VFA.

The exact growth rate of the biobased chemicals industry will depend on a number of factors. The relative prices of oil and agricultural raw materials, combined with the speed of technological progress, will be major determinants for switching from fossil to renewable feedstocks.

The US market analysis company MarketsandMarkets foresees that the industry for renewable chemicals will be growing rapidly in the coming years. They estimate that the global market for renewable chemicals will increase from USD 57 billion in 2013 to USD 83.4 billion by 2018, delivering an annual growth rate of 7.7% during the period.

California biotech company Rennovia Inc. is more optimistic regarding relative growth, as they anticipate the global market for renewable chemicals to grow approximately three times during the coming five years! But Rennovia's starting point is a modest USD 3.6 billion of today's market, growing to around USD 12 billion by 2020. The background for such differences is uncertain, but it underlines the need for a critical position and common sense when looking into market forecasts.

Traditional players in the market for chemicals might enter the market for sustainable products by either buying or joining in strategic partnerships with small start-up companies. These start-ups can offer a mature technology platform but have a business plan that lacks, for example, capital or distribution and consumer networks.

Large industrial companies from other sectors that have specific demands for chemicals or technologies may well team up with a biotech company offering exactly this product. For example, a strategic partnership has been established between soft drinks manufacturer The Coca-Cola Company, Austrian ALPLA GmbH that manufactures plastic containers, and the company Avantium to develop a polyethylene furanoate (PEF) recyclable plastic bottle made from plant biomass through their native fermentation-free catalytic synthesis technique YXY technology, which meets the beverage company's requirements and specifications for soft drink bottles.

Although there are prominent examples of biotechnology companies producing chemicals at industrial scale that are derived from plant biomass or other renewable feedstocks, most activity is still at the R&D stage, and this also applies to VFA. Currently, there are a number of short chain fatty acids, which are produced at a larger scale from renewable sources by microbial conversion and many of these are used for polymer plastic applications. Companies featuring established processes and in-house developed technologies are listed in Table 3.

The Dutch company Avantium exploits carbohydrates from plant biomass to produce polyethylene furanoate, a 100% recyclable plastic material featuring improved properties. The carbohydrates are converted to PEF by the company's YXY catalysis technology. They also use the side-streams that are produced when lignocellulosic biomass is pretreated

Table 3: Biotech companies producing organic acids mainly from fermentation of renewable feedstocks.

Company, reference	Products	Technology platform
Avantium, N.L https://www.avantium.com/	Acids and fuels PEF plastic	Lignocellulosic feedstocks YXY catalysis
BioAmber, Canada https://www.bio-amber.com/	Succinic acid	Fermentation of corn syrup
BioSyntha Technology Ltd., UK http://www.biosyntha.com/	Novel acids and fuels	Plant biomass feedstock syngas fermentation with engineered strains
Cargill, USA http://www.cargillfoods.com/	Citric acid	Carbohydrate fermentation
Genomatica, USA http://www.genomatica.com/	Adipic acid	Sugar and syngas fermentation
LanzaTech, NZ http://www.lanzatech.com/	Acetic acid fuels	Waste gasses feedstocks fermentation hybrid separation
Metabolic Explorer, France http://www.metabolic-explorer.com/	Butyric acid PDO MPG methionine	Second-generation biomass feedstocks and fermentation
Myriant Corporation, USA http://www.myriant.com/	Lactic acid succinic acid	Plant biomass feedstock fermentation
NatureWorks, USA http://www.natureworksllc.com/	Lactic acid	Carbohydrate fermentation
Rennovia Inc., USA http://www.rennovia.com/	Adipic acid hexamethylenediamine	Plant biomass feedstock chemical catalysis
Reverdia, Netherlands http://www.reverdia.com/	Succinic acid	Yeast fermentation of starch
Succinity, Germany http://www.succinity.com/	Succinic acid	Bacterial fermentation of biomass
Verdezyne Inc., USA http://www.verdezyne.com/	Dodecanedioic acid adipic acid sebacic acid	Plant biomass feedstock fermentation using engineered yeast
Zeachem Inc., USA http://www.zeachem.com/	Acetic acid ethanol ethyl acetate	Plant biomass feedstock fermentation

for the recovery of carbohydrates. 5-Hydroxymethylfurfural (HMF) and furfural are two platform chemicals that can be obtained from the dehydration of C6 and C5 sugars. They can be further converted into furanic derivatives such as 2,5-furandicarboxylicacid (FDCA) or furfuryl alcohol (FA) [112], which are precursors to biobased polymers.

British BioSyntha Technology Ltd. is R&D company that develops and sells pilot scale fermentation technology with wild-type or engineered strains. Their fermentation technology produces novel chemicals and novel fuels from gasification of waste plant biomass and other renewable resources.

The New Zealand company LanzaTech produces acetic acid and fuels from microbial conversion of carbon monoxide waste gasses from various sources. Their core technology is the productive microbe used and their separation technology [113]. According to the company they are about to take products from demonstration scale to commercialization and market introduction.

The US company Myriant develops technology for production of lactic and succinic acids. Their business model is based on partnerships and licensing for commercial production: for example, production of lactic acid via fermentation where their Spanish partner Purac has licensed Myriant's process to produce lactic acid and has been producing the chemical on a commercial scale since 2008.

The French company Metabolic Explorer (METEX) produces acids such as butyric acid and alcohols from 2nd-generation biomass. In 2010 the company announced their first industrial pilot phase and validation of PDO and has inaugurated a manufacturing plant in Malaysia. The US based Rennovia Inc. is working on development, scale-up, and commercialization of an array of chemical products from renewable feedstocks coupled with traditional catalysis technology. The company's products are, for example, adipic acid and hexamethylenediamine (HMD), which are building block chemicals of commercial importance.

Verdezyne Inc., a US based company, develops technologies for production of organic acids such as dodecanedioic acid, adipic acid, and sebacic acid with broad applications. In December 2014 the company announced an agreement with Malaysian partner Bio-XCell to construct and run Verdezyne's first commercial-scale renewable manufacturing facility. The product is dodecanedioic acid.

Table 4: Global market and prices of acetic acid.

Year	Production[1] (t)	Price[2] (USD/t)	Reference
2000	$8.3 \cdot 10^6$		[22]
2008	$1 \cdot 10^7$		[115]
2014	$1.5 \cdot 10^7$		http://www.essentialchemicalindustry.org/
2014		500–850	http://www.alibaba.com/
2015	$1.6 \cdot 10^7$		http://www.lanzatech.com/

[1]Data are supplied either as actual production from petrochemistry or as production capacity.
[2]Price is purity and quantity dependent. Free On Board (FOB).

Table 5: Global market and prices of propionic acid.

Year	Production[3] (t)	Price[4] (USD/t)	Reference
1992	$1 \cdot 10^5$		[116]
1996	$1.8 \cdot 10^5$		[22]
1997	$1.9 \cdot 10^5$		[22]
1999	$2 \cdot 10^5$		[22]
2006	$1.3 \cdot 10^5$[5] $3.5 \cdot 10^5$ $3.8 \cdot 10^5$		[117] http://www.marketsandmarkets.com/ [118]
2012		1000	[119]
2014	$3.8 \cdot 10^5$	1500–2000 1600–2300	[120] http://www.lookchem.com/ http://www.alibaba.com/

[3]Data are supplied either as actual production from petrochemistry or as production capacity.
[4]Price is quality and quantity dependent. Free On Board (FOB).
[5]In 2006, a minor fraction was produced by fermentation and commercialized for food and fragrance manufacturing [117].

The US based Zeachem Inc. has a demonstration plant facility in Boardman, Oregon, inaugurated in 2012, that produces ethanol and acetic acid from plant biomass feedstock in a hybrid process. Their product capacity for bioacetic acid is currently almost 100,000 L/year. The company has initiated a commercial scale plant to open at the same location with a capacity of nearly 100 million L/year. ZeaChem utilizes a hybrid process consisting of microbial and thermochemical processing in combination, which yields C2 products such as ethanol and acetic acid and derivatives. After pretreatment of the biomass, sugar streams are fermented by homofermentative, thermophilic anaerobes to acetic acid without any microbial CO_2 production [114]. Acetic acid is then subjected to esterification and the resulting ester is combined with hydrogen to produce ethanol. The hydrogen required to convert the ester to ethanol is derived from syngas produced by gasification of the lignin fraction from the biomass feedstock. The remainder of the syngas is combusted to create steam and power for the process. With process adjustments, the technology can produce three-carbon products including propionic acid, ethyl propionate, propanol, and propylene according the company's own information.

6. VFAs Prices and Volumes

Unless clearly stated, all data about prices and volumes supplied in Tables 4, 5, and 6 are based on petrochemical production routes.

Table 6: Global market and prices for butyric acid. In 2006, a minor fraction was produced by fermentation and commercialized for food and fragrance manufacturing [117].

Year	Production[6] (t)	Price[7] (USD/t)	Reference
2008	$5 \cdot 10^4$		[124]
2011	$5 \cdot 10^5$		[125]
2014		1800–1900	http://www.alibaba.com/

[6]Data are supplied either as actual production from petrochemistry or as production capacity.
[7]Price is quality and quantity dependent. Free On Board (FOB).

7. Acetic Acid

According to the market analysis company IHS (2013 figures), a 4-5% growth per year of the global market is expected. Growth will be driven mainly by the Chinese market with a rapid expansion in production facilities and a future consumption growth of acetic acid is expected to be around 7% per year. While the primary application of acetic acid is within the food industry, the second largest global acetic acid use will become production of terephthalic acid (TPA). TPA is mainly used for the manufacture of polyethylene terephthalate (PET) packaging fibers, clothing, plastic bottles, and films. A similar global volume of acetic acid will be used for acetate esters that are exploited mainly as solvents for inks, paints, and coatings.

The US company Celanese and British Petroleum (BP) are among top ranked companies in the world regarding acetic acid production, which is currently produced from oil-derived methanol and carbon monoxide using a chemical catalyst. Therefore, the prospect for growth in sustainable production of acetic acid is dependent upon the nature of feedstocks and processes for methanol production. According to Green Chemicals blog, the total global revenue for biobased production of acetic acid and derivative ethyl acetate amounts to USD 21 billion.

8. Propionic Acid

The German company BASF is the largest propionic acid manufacturer in the world and produces $8.8 \cdot 10^4$ tons per year in Germany and China. The acid, which is produced via the petrochemical route, is used in products for feed grain preservation under their trade names Luprosil®, containing propionic acid, and Lupro-Grain®, containing ammonium propionate salt [121]; both products are claimed to reduce CO_2 emission because drying is not necessary when the grain is conserved. Other major industrial manufacturers, such as US company The Dow Chemical Company, maintain traditional production processes while at the same time developing sustainable production in order to cut production expenses and narrow the gap between fermentation and petrochemical processes [122].

While the market price for propionic acid from the petrochemical route was around 1000 USD/ton in 2012 [119], the price for the acid from the biotechnological route was about 1500–2000 USD/ton. Today propionic acid prices are around 1600–2000 USD/ton while the calcium or sodium salts are slightly cheaper per metric ton. Globally, the use of propionic acid dominates the large market for feed preservatives. The principal use of propionic acid is as an acidifier for animal feed, grain, and food where calcium and sodium propionates accounted for 78.5% of world propionic acid consumption in 2012, according to IHS. Other fast-growing markets include propionate esters such as n-butyl and pentyl propionate because these esters are increasingly being used as replacements for solvents listed as hazardous air pollutants according to IHS. The global market in terms of revenue was estimated to be worth USD 935.7 million in 2012 and is expected to reach USD 1.7 billion by 2018 according to MarketsandMarkets and to grow at a rate of 7.8%–9.6% from 2013 to 2018. While Europe and the US accounted for around two-thirds of the global consumption in 2012, emerging markets such as Asia and Africa are likely to be responsible for future growth in production and use of propionic acid and derivatives.

The major growth in propionic acid demands within a single market niche is the use as an additive to prolong shelf life of preserved food. Private consumers are increasingly demanding "natural" and healthier food additives and propionic acid is "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration (FDA). Growth in demands for propionic acid is also attributed to the growing demands of organic food products mainly in North America and Western Europe. The trend in natural preservatives originated from European nations wishing to market the clean label food products free of artificial additives especially for preservation of organic foods [123]. An example is the company Danisco's food preservative MicroGard® which is based on propionic acid and marketed as a natural and safe antimicrobial compound.

Thus, changing lifestyles and the fast growth in convenience foods and beverage industry have increased the demand for natural preservatives with expected direct effects on propionic acid demand. By 2016 the global food preservatives market is estimated to reach revenues of USD 2.6 billion, growing at a rate of 2.5% in the coming years thus supporting fast growth amongst "natural" preservatives. Consequently, the future demand for propionic acid is strongly dependent on both food and feed production.

9. Butyric Acid

Eastman Chemical Company is a manufacturer of butyric acid via their Oxo Low-Pressure Technology and is one of the major global players in the market. As Eastman foresaw continuous market growth, they expanded their butyric acid production facility in Newport, Tennessee, in 2013/2014 by around 7000 tons/year.

10. From Promise to Market

Lignocellulosic biomass can be converted into more than a hundred different chemicals [126]. Among them are new chemicals but also established compounds with immediate drop-in features, such as the VFAs, that can directly substitute for fossil-derived chemicals and constitute platform chemicals, monomers, chemical intermediates, or end products in many industrial sectors.

Because of their functionality (chemical reactivity) and natural origin, the market for acetic, propionic, and butyric acids is already huge today and world market demands for these acids are predicted to grow in the coming years. Although the demand for some chemicals will expire and their use will cease, other chemicals will continue to be in demand, for instance, as platforms and materials but also as liquid transportation fuels. Despite that chemicals and materials can be produced from lignocellulosic biomass by pretreatment and fermentation processes, there are still scientific obstacles in order for biotechnology to become the principal technology for sustainable production of VFAs and other biochemicals within the chemical industry.

The European Technology Platform For Sustainable Chemistry [127] is a joint venture between Cefic and several European chemical organisations that recently published a strategic innovation and research agenda [127], which states that the main obstacle to the spread of biobased chemicals is the supply of sufficient amounts of biomass that are price competitive, and to ensure a stable supply of 2nd-generation biomass, which does not compete with food or feed production. SusChem highlight three areas, which they consider major R&D topics in the development

of biobased chemicals: (1) fractionation of biomass into its components and improved pretreatment methods for lignin conversion and (2) development of robust industrial microbial fermentation strains with tailored capabilities such as improved resistance to their own metabolic products and (3) process developments, which combine green chemistry and biotechnology technologies for improvement of biomass utilization and improved economics.

But besides the technical challenges, there are also economic challenges and issues about expenditure and costs are without any doubt of great importance to facilitate transition from an oil-based to a biobased economy. It will require a deliberate and sustained focus on biomass valorization, microbial productivity, and improved processes to reduce total costs of biological VFA production. Costs reduction is feasible: for example, production costs of biosuccinic acid were reduced to 25% over twelve years by keeping economics in focus [128].

As shown in this review, biobased products and materials are structurally identical to those obtained from fossil-based feedstocks and there is a potential to develop new biobased products and materials that cannot be produced from fossil feedstocks. To become truly competitive, biochemicals and biomaterials should, however, be genuinely new or feature improved properties and economics compared to fossil-based products [129].

There are without doubt still technical challenges to be solved [130] before a full scale commercialization of microbial processes for production of renewable chemicals has been marketed and a wide range of biomass-derived biochemicals are available on the global market. On the other hand, the outlook for biotechnology is promising because there has never been a period in the history of biotechnology where interests [131] and needs have been more obvious than presently.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Synthesis of Novel Pyrimethanil Grafted Chitosan Derivatives with Enhanced Antifungal Activity

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In this study, three pyrimethanil grafted chitosan (PML-g-CS) derivatives were obtained. The structures of the conjugates were confirmed by FT-IR, ^{1}H NMR, and EA. The grafting ratios were measured by HPLC. Antifungal properties of pyrimethanil grafted chitosan (PML-g-CS) derivatives against the plant pathogenic fungi *Rhizoctonia solani* and *Gibberella zeae* were investigated at concentrations of 100, 200, and 400 mg/L. The PML-g-CS derivatives showed enhanced antifungal activity in comparison with chitosan. The PML-g-CS-1 showed the best antifungal activity against *R. solani*, whose antifungal index was 58.32%. The PML-g-CS-2 showed the best antifungal activity against *G. zeae*, whose antifungal index was 53.48%. The conjugation of chitosan and pyrimethanil showed synergistic effect. The PML-g-CS derivatives we developed showed potential for further study and application in crop protection.

1. Introduction

Chitosan (CS), a kind of natural cationic polysaccharides, consists of β-1,4-linked glucosamine with various N-acetyl glucosamine residues [1]. It is naturally renewable and exhibits a lot of special properties like biocompatibility, biodegradability, and bioactivity. Due to its special chemical structure and characteristics, chitosan is widely used in many areas such as food industry, medical cares, and agriculture [2]. Particularly, it was proved to have a broad-spectrum antifungal activity against a variety of fungi. Allan and Hadwiger found that, of the 46 tested fungi, chitosan had antifungal activity against 32 isolates [3]. Bautista-Baños et al. described the fungicidal activity of chitosan on *Colletotrichum gloeosporioides* in vitro stating that 3% chitosan can completely inhibit its growth [4]. However, chitosan also shows some demerits for its further application; the most important limit is that its antifungal effect is weaker than many commercial fungicides. Thus, there have been a lot of methods to enhance its antifungal effect [5]. One of the approaches is the chemical modification of chitosan. A wide range of bioactive groups were combined to the chitosan molecular structure to achieve different chitosan derivatives such as carboxymethyl chitosan [6], sulfate chitosan [7], chitosan quaternary ammonium salt [8], and hydroxypropyl chitosan [9].

Recently, the synthesis and property study of chitosan conjugates has received increased attention. Different kinds of molecules are grafted onto chitosan chains by chemical bond [10]. The complexation of chitosan and metal ions enhanced its antibacteria activity [11, 12]. The conjugation by grafting of antioxidant molecules onto chitosan improves the antioxidant activity [13]. However, little attention has been paid to the improvement of chitosan antifungal activity by the conjugation of antifungal molecules [14, 15].

Pyrimethanil (PML) is a kind of traditional fungicide widely used in agriculture [16]. Its commercial product name is "Scale". It occupies great share in commercial fungicide market due to its high-efficiency and special mechanism [17].

Pyrimethanil has excellent antifungal effect on grey mould of grapes and strawberries. It inhibits the growth of *Monilia laxa* and *Drepanopeziza ribis* observably. The antifungal mechanism of pyrimethanil is to inhibit the secretion of fungi infecting enzyme. It has both protecting and treating function.

In order to improve the antifungal activity of chitosan, we did our research on the conjugation of chitosan and pyrimethanil technical material. In our study, pyrimethanil was grafted onto the chitosan polymer chains for the first time. The synthesized pyrimethanil grafted chitosan derivatives were characterized by Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR), and element analysis (EA) to confirm the conjugation. The grafting ratios were detected by HPLC. The antifungal activities of the compounds were also determined.

2. Experiment

2.1. Materials and Reagents. Chitosan (86.3% degree of deacetylation) was purchased from Qingdao Yunzhou Biochemical Corp. Pyrimethanil technical material (purity higher than 96%) was purchased from Yantai Kechuang Chemical Corp. Chloroacetyl chloride was purchased from Sinopharm Chemical Reagent Co., Ltd. Chloropropionyl chloride and 4-(chloromethyl) benzoyl chloride were purchased from Aladdin Chemical Reagent Co., Ltd. Isopropanol, dichloromethane, trichloromethane, potassium carbonate, glacial acetic acid, methanol, sodium hydroxide, and tween 80 were purchased from Sinopharm Chemical Reagent Co., Ltd. and were all of analytical grade. Methanol, sodium acetate, and acetic acid for HPLC analysis were purchased from Merck Drugs & Biotechnology and were of chromatographic grade.

2.2. Analytical Methods. Fourier transform infrared (FT-IR) spectra range within the 4000–400 cm^{-1} regions on a Thermo Scientific Nicolet iS10 FT-IR spectrometer with attenuated total reflection intelligent components. ^1H NMR (nuclear magnetic resonance) spectra were investigated on a JEOL JNM-ECP600 spectrometer; solvents were CD_3COOD and D_2O. The elemental analysis (C, H, and N) was performed using a Vario EL-III elemental analyzer. The percentages of carbon, nitrogen, and hydrogen were estimated. The grafting ratios of pyrimethanil grafted chitosan derivatives were determined by an Agilent 1260 HPLC (Agilent Technologies, USA) equipped with a UV-detector. Chromatography was performed on C18 reversed-phase column.

2.3. Synthesis of Pyrimethanil Grafted Chitosan Derivatives. The synthesis was carried out by two steps. For the first step, pyrimethanil technical material was modified by chloroacetyl chloride, chloropropionyl chloride, and 4-(chloromethyl) benzoyl chloride to achieve a series of intermediates for the following reaction. The second step was the synthesis of pyrimethanil grafted chitosan derivatives [18].

In the first step, pyrimethanil (4 g) was dissolved in dichloromethane (l80 mL). After that, 2 times the equivalent amount of chloroacetyl chloride (chloropropionyl chloride or 4-(chloromethyl) benzoyl chloride) was added into the dichloromethane in drops in an ice bath. 1.2 times of equivalent amount of potassium carbonate was also added into the reaction system. The end of reaction was tested by thin layer chromatography (TLC). When the reaction was over, the reaction mixture was added into water (50 mL) to remove extra acyl chloride. The reaction mixture was poured into a separating funnel. The organic phase at the lower layer was collected and evaporated to dryness to achieve the intermediates.

In the second step, purified chitosan (3 g) was dispersed in trichloromethane (50 mL). After 30 minutes of magnetic stirring at room temperature, 12 mL of aqueous NaOH (10 M) was added to the suspension and the alkalization lasted for 4 hours. The reaction proceeded to 4 h at temperature of 50°C and the solid product was then filtered, suspended in 150 mL of methanol, and neutralized with glacial acetic acid. The product was extensively washed with 100% methanol and dried at room temperature. Finally, the pyrimethanil grafted chitosan derivatives were obtained.

2.4. Determination of Weight Average Molecular Weight of Pyrimethanil Grafted Chitosan Derivatives. The weight average molecular weight (Mw) of chitosan and PML-g-CS derivatives were measured by gel permeation chromatography using Agilent 1260 HPLC (Agilent Technologies, USA) equipped with a refractive index detector. Chromatography was performed on TSK G5000-PWXL column, using 0.2 M CH_3COOH/0.1 M CH_3COONa aqueous solution as mobile phases at a flow rate of 1.0 mL/min with column temperature at 30°C. The sample concentration was 0.4% (w/v). The standards used to calibrate the column were dextrans samples with Mw of 1,300,000, 670,000, 270,000, 80,000, 50,000, and 25,000 Da (Sigma, USA).

2.5. Determination of Grafting Ratios of Pyrimethanil Grafted Chitosan Derivatives. 0.1 g PML-g-CS was added into methanol solution (100 mL, pH 4.0). Hydrolysis lasted 2 hours to extricate all the pyrimethanil from the conjugates. Then, the solution was diluted to 10 times for detection. The determination of grafting ratio of PML-g-CS derivatives was measured by an Agilent 1260 HPLC (Agilent Technologies, USA) equipped with a UV-detector. Chromatography was performed on C18 reversed-phase column, using H_2O/methanol (15 : 85) solution as mobile phases at a flow rate of 1.0 mL/min with column temperature at 30°C. The testing wave length was set to 220 nm [19]. The standards used to calibrate the results were pyrimethanil dissolved in methanol with a series concentration of 0.01, 0.02, 0.04, 0.08, and 0.1 mg/mL. The grafting ratios were calculated based on the standard curve.

2.6. Antifungal Activity Assay. Antifungal assays against *Rhizoctonia solani* CGMCC 3.28 and *Gibberella zeae* CGMCC3.42 were evaluated in vitro according to the agar medium method described in literature [20]. Chitosan and PML-g-CS derivatives were dissolved in 0.5% (v/v) acetic

acid at an original concentration of 1% (w/v). The solutions were mixed with sterile molten potato dextrose agar (PDA) to obtain final concentrations of 100 mg/L, 200 mg/L, and 400 mg/L.

The antifungal index was calculated by the following formula:

$$\text{Antifungal Index } (\%) = \frac{D_{\text{blank}} - D_{\text{average}}}{D_{\text{blank}} - D_{\text{fungus}}} \times 100. \quad (1)$$

D_{blank} referred to the diameter of the blank plate without fungus inoculated. D_{average} referred to the average diameter of the fungal colony. D_{fungus} referred to the diameter of the inoculated fungal disk.

2.7. Statistical Analysis. Antifungal bioassay experiments were performed in triplicate. The data were analyzed with Origin, version 8.0 (Origin Lab Corp., Northampton, MA), and all the results were expressed as the mean ± SD. Results with $P < 0.05$ were considered to be statistically significant.

3. Results and Discussion

3.1. Preparation of Reaction Intermediates and Pyrimethanil Grafted Chitosan Derivatives. In this study, pyrimethanil was grafted onto chitosan using three kinds of acyl chlorides as linkers. The grafting reaction was carried out by two steps: the synthesis of intermediates and the synthesis of PML-g-CS derivatives. The reaction process for the conjugation of pyrimethanil grafted chitosan derivatives was proposed in Figure 1. For the first step, three kinds of reaction intermediates were synthesized by mixing pyrimethanil and acyl chlorides in dichloromethane. The acid-binding agent was potassium carbonate. The end of reaction was tested by thin layer chromatography (TLC). The developing solvent of the TLC process was methanol and petroleum ether (v : v = 1 : 5). For the second step, PML-g-CS derivatives were obtained. We named the conjugates whose linkers were chloroacetyl chloride, chloropropionyl chloride, and 4-(chloromethyl) benzoyl chloride as PML-g-CS-1, PML-g-CS-2, and PML-g-CS-3, respectively.

The graft ratios measured by HPLC for three PML-g-CS derivatives were 8.13%, 6.54%, and 5.05%. The standard curve was $y = 106179x - 1116.2$, and $R^2 = 0.992$.

3.2. Characterization of Pyrimethanil Grafted Chitosan Derivatives

3.2.1. FT-IR Spectra. Figure 2 showed FT-IR spectra of chitosan, PML-g-CS-1, PML-g-CS-2, and PML-g-CS-3. For chitosan, the broad band around 3384 cm^{-1} was attributed to OH and NH stretching vibration. The weak band at 2879 cm^{-1} was the characteristic absorbance peak of CH. The band at 1595 cm^{-1} was assigned to the N-H bending of the primary amine. The bands at 1650, 1550, and 1320 cm^{-1} were attributed to the C-O stretching, N-H bending, and C-N stretching of the residual N-acetyl groups, respectively. For pyrimethanil, the typical absorption peaks between 1500 and 1600 cm^{-1} were due to the C-N band and benzene ring [21]. For PML-g-CS-1 and PML-g-CS-2, the obvious absorption peak at 1750 cm^{-1} was attributed to bending vibration of C-H which belongs to the previous acyl chloride structure. For PML-g-CS-3, new bands at 700 cm^{-1} and 800 cm^{-1} were observed. Those C-H bands' absorption peaks indicated a monosubstituted benzene ring and a p-substituted benzene ring, which fitted the theoretical structure of PML-g-CS-3. All of the above results exhibited that pyrimethanil grafted chitosan derivatives had been successfully prepared.

Table 1: The weight average molecular weight of chitosan and pyrimethanil grafted chitosan derivatives.

Samples	Mw (kDa)	Degree of dispersity
Chitosan	1058.6	1.28
PML-g-CS-1	1057.5	1.35
PML-g-CS-2	1053.4	1.17
PML-g-CS-3	1055.2	1.32

3.2.2. ^1H NMR Spectra. The ^1H NMR spectra of chitosan, PML-g-CS-1, PML-g-CS-2, and PML-g-CS-3 were shown in Figure 3. As shown in Figure 3, a series of peaks belonging to chitosan was detected: H-1 signal was the single peak at 4.4 ppm, H-2 peak was at 2.9 ppm, and peaks of H-3 to H-6 were the multiple peaks from 3.3 ppm to 3.7 ppm. For PML-g-CS derivatives, the characteristic absorption peaks of pyrimidine group were at 1.8 ppm and 5.6 ppm. The absorption peaks at 1.0 ppm and 3.3 ppm were attributed to chloroacetyl chloride and chloropropionyl chloride parts as linkers in PML-g-CS-1 and PML-g-CS-2, respectively. The absorption peaks of benzene protons of PML-g-CS-3 at 5.1 ppm were observed. All the characteristic absorption peaks of each derivative were marked in Figure 3 separately. Consistent with the FT-IR results above, the structure of PML-g-CS derivatives was further confirmed by ^1H-NMR.

3.2.3. Gel Permeation Chromatography. Taking into account that the antifungal activity of chitosan is dependent upon the molecular weight, we calculated the weight average molecular weight of chitosan and PML-g-CS derivatives by GPC. The results were shown in Table 1. The degree of dispersity was between 1.1 and 1.4, showing that the results were measured validly. The GPC results showed that the Mw of chitosan and PML-g-CS derivatives did not change in statistics. In such condition, the average molecular weight did not have an impact on the antifungal activity results.

3.2.4. Elemental Analysis. Elemental analysis results of PML-g-CS derivatives are shown in Table 2. The degree of substitution (DS) of PML-g-CS derivatives was calculated on the basis of the percentage. As shown in Table 2, the DS of PML-g-CS derivatives were 8.45%, 6.72%, and 5.18%, which matched the results calculated by the HPLC method.

3.3. Antifungal Activity of Pyrimethanil Grafted Chitosan Derivatives. Antifungal properties of pyrimethanil grafted

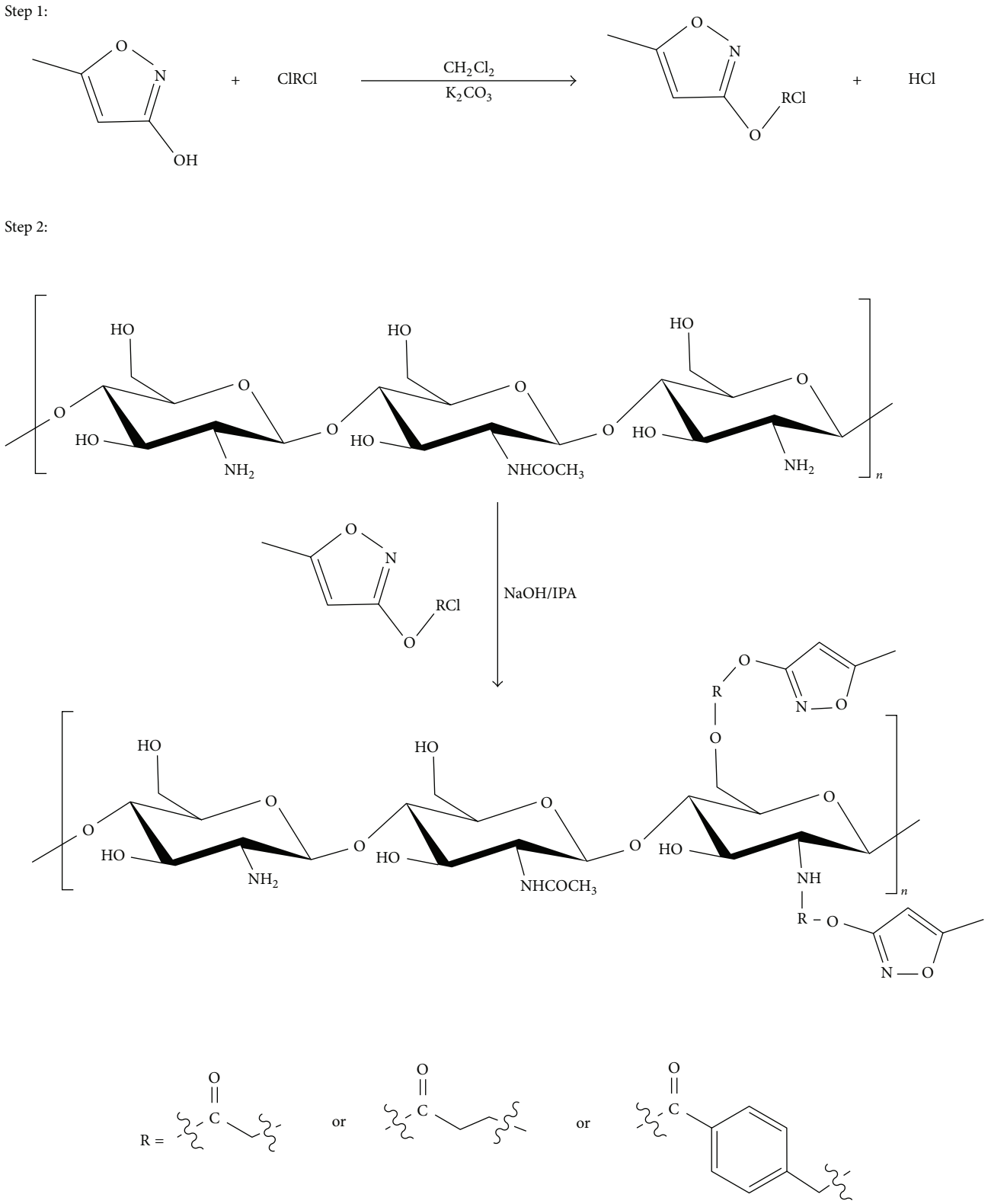

Figure 1: The reaction process for the PML-g-CS derivatives.

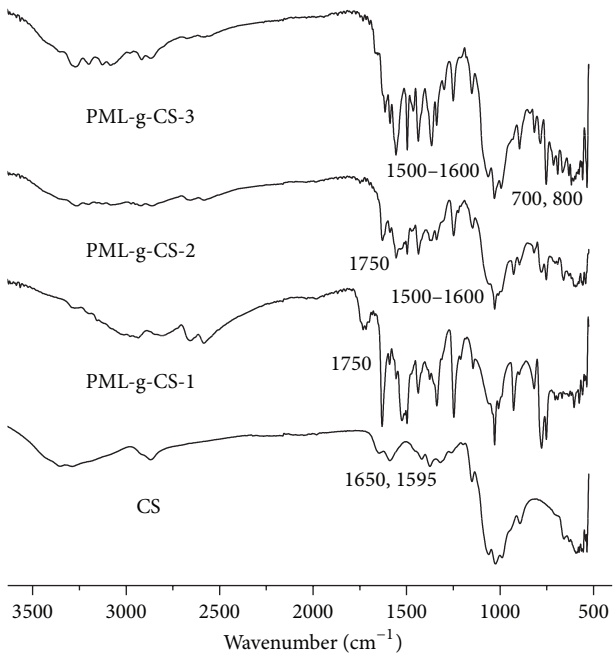

Figure 2: FT-IR spectra of chitosan and PML-g-CS derivatives.

Table 2: Elemental analysis results and degree of substitution of chitosan and pyrimethanil grafted chitosan derivatives.

Samples	Elemental analysis (%)			Degree of substitution (%)
	C	N	H	
Chitosan	40.05	7.29	6.41	—
PML-g-CS-1	40.16	8.06	6.24	8.45
PML-g-CS-2	40.28	7.96	6.22	6.72
PML-g-CS-3	40.10	7.75	6.13	5.18

chitosan (PML-g-CS) derivatives against the plant pathogenic fungi *R. solani* and *G. zeae* were investigated at concentrations of 100, 200, and 400 mg/L.

The antifungal activities of chitosan and chitosan derivatives are influenced by various factors such as molecular weight, degree of deacetylation, sample concentration, and pH value [22]. In this study, chitosan and pyrimethanil grafted chitosan (PML-g-CS) derivatives were dissolved in 0.5% (v/v) acetic acid at an initial concentration of 1% (w/v). The sample solutions were all diluted with sterile molten potato dextrose agar (PDA). The addition of the acetic acid might contribute to the antifungal effect. So the inhibitory activity of different concentrations of acetic acid aqueous solution against *R. solani* and *G. zeae* was evaluated. The testing concentrations were set corresponding to the investigated concentrations of samples. As shown in Figure 4, the inhibitory activity of acetic acid on the selected fungi was influenced by the concentrations. At concentrations lower than 0.02% (corresponding to 400 mg/L of the sample), acetic acid aqueous solution had no inhibitory effect on *R. solani* and *G. zeae*. The results showed that, at concentrations ranging from 100 to 400 mg/L, the diluted acetic acid had no antifungal activity. When the concentrations were higher than 0.02%, acetic acid would inhibit the growth of *R. solani* and *G. zeae*. It was shown that 0.05% acetic acid aqueous solution inhibited growth of *R. solani* at 10.5%.

R. solani is a pathogen which causes diseases on paddies, peanuts, sesame, and so on [23]. As shown in Figure 5, all PML-g-CS derivatives exhibited much better antifungal activity compared with the original chitosan. The antifungal activity was enhanced with the increasing of concentration. The antifungal indexes of PML-g-CS derivatives ranged from 42.85% to 58.32% at 400 mg/L, while the indexes of chitosan only reached by 26.68%. The inhibitory activity followed a sequence of PML-g-CS-1 > PML-g-CS-2 > PML-g-CS-3 > chitosan. Different linkers also impacted the antifungal activity. The chloroacetyl chloride as linker was better than chloropropionyl chloride and 4-(chloromethyl) benzoyl chloride. This may be due to two reasons. One reason is that the smaller linker led to easier combination with the action sites. The other is that the grafting ratio is the highest when using chloroacetyl chloride as linker. Therefore, more pyrimethanil was on the polymer chain.

G. zeae is a pathogen mainly infecting gramineous plants, such as corn and wheat [24]. Table 2 represents the antifungal results of the PML-g-CS derivatives against *G. zeae*. Compared with chitosan, antifungal activity of PML-g-CS derivatives was enhanced. But the degree of improvement was not as obvious as the effect against *R. solani*. The antifungal activity of the original chitosan against *G. zeae* was stronger than *R. solani*, while the antifungal activity of PML-g-CS derivatives against *G. zeae* was weaker than *R. solani*. The antifungal index of PML-g-CS-3 at 400 mg/L was 27.9%, which was lower than the index of chitosan at the same concentration (28.72%). The results indicated that the modification by grafting pyrimethanil onto chitosan did not obviously enhance the antifungal activity against *G. zeae*. *G. zeae* was not sensitive to the new compounds.

By analyzing the abovementioned results, it was found that the DS indexes greatly influenced the antifungal activity of all PML-g-CS derivatives. The DS indexes of three derivatives changed with different linkers used in the synthesis process. The higher DS meant more pyrimethanil was grafted to the chitosan structure. What is more, the grafted pyrimethanil group contributed a lot to the improvement of antifungal activity of the PML-g-CS derivatives. The DS followed a sequence of PML-g-CS-1 > PML-g-CS-2 > PML-g-CS-3; meanwhile, the antifungal activity had the same results against both *R. solani* and *G. zeae*.

As shown in Table 3, the antifungal activity of pyrimethanil against *R. solani* and *G. zeae* was much stronger than the antifungal activity of PML-g-CS derivatives. At concentrations higher than 100 mg/L, the inhibition indexes of pyrimethanil technical material against *R. solani* and *G. zeae* were both 100%, while the highest inhibition effect of PML-g-CS derivatives was 58.32% and 53.48%.

When the concentrations of PML-g-CS derivatives were 100, 200, and 400 mg/L, considering the grafting ratios, the content of pyrimethanil was about 8, 16, and 32 mg/L, respectively. At the equivalent concentration, the antifungal activity of PML-g-CS derivatives was stronger than pyrimethanil

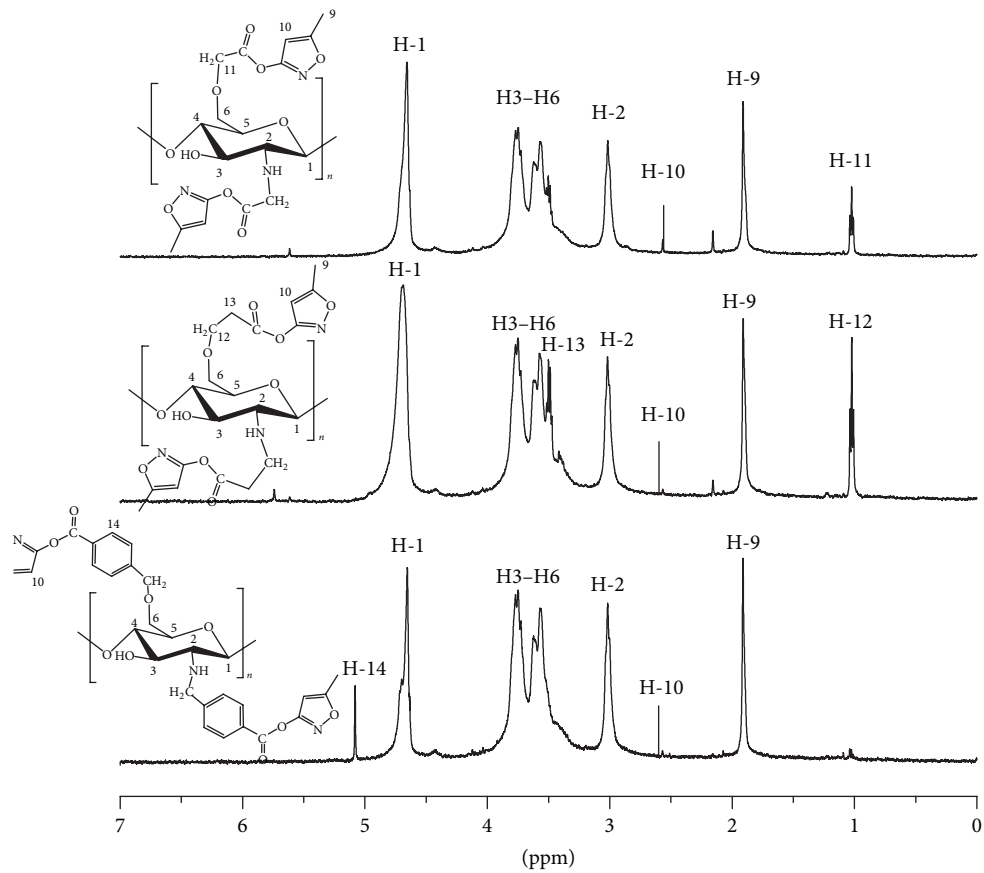

Figure 3: ^{1}H-NMR spectra of chitosan and PML-g-CS derivatives.

Table 3: Antifungal activity of chitosan and pyrimethanil grafted chitosan derivatives at different concentrations.

Samples	Concentrations (mg/L)	Antifungal index (%)	
		R. solani	*G. zeae*
Chitosan	100	12.13	15.55
	200	17.82	20.8
	400	26.68	28.72
PML-g-CS-1	100	41.49	27.58
	200	49.75	36.5
	400	58.32	48.9
PML-g-CS-2	100	30.23	37.26
	200	36.23	44.59
	400	48.4	53.48
PML-g-CS-3	100	32.47	25.85
	200	35.09	34.5
	400	42.85	38.9
Pyrimethanil	8	29.45	27.9
	16	33.75	32.35
	32	38.2	37.15
	100	100	100
	200	100	100
	400	100	100

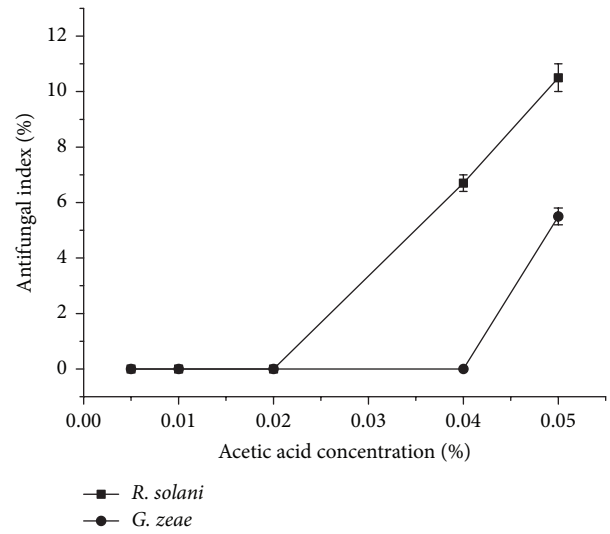

Figure 4: Inhibitory effect of different concentrations of acetic acid aqueous solution.

technical material. This meant that the conjugation of chitosan and pyrimethanil showed synergistic effect. It was an efficient method to enhance the antifungal activity of chitosan.

Based on the bioassay results above, it was found that pyrimethanil grafted chitosan (PML-g-CS) derivatives had enhanced antifungal activity in comparison with chitosan.

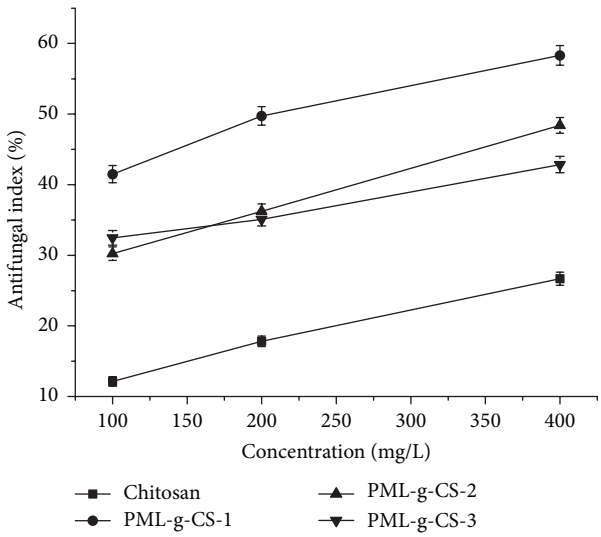

Figure 5: Antifungal activity of PML-g-CS derivatives against *R. solani*.

The PML-g-CS-1 derivative showed the best effect of three. The graft of pyrimethanil onto chitosan chain could improve the antifungal activity of chitosan in general. Further research may force the increase of antifungal activity to achieve a more economical product.

4. Conclusion

The preparation, characterization, and antifungal activity of pyrimethanil grafted chitosan (PML-g-CS) derivatives were investigated in the present study. Results suggested that the pyrimethanil could be grafted onto chitosan by using different acyl chlorides as linkers. In addition, the conjugation was confirmed by FT-IR and EA. Antifungal assays of chitosan and PML-g-CS derivatives against *R. solani* and *G. zeae* were evaluated in vitro. Statistical analysis also indicated that antifungal activity of chitosan was enhanced by grafting with pyrimethanil. Our data provided a novel approach for the modification of chitosan and indicated pyrimethanil grafted chitosan derivatives could be explored as promising antifungal agents.

Competing Interests

The authors do not have any conflict of interests regarding the content of the paper.

Acknowledgments

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Research Article

Plant Growth Biostimulants Based on Different Methods of Seaweed Extraction with Water

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We explored two methods for obtaining aqueous extracts: boiling and soaking of Baltic seaweeds (EB and ES, resp.). Algal extracts were characterized in terms of polyphenols, micro- and macroelements, lipids content, and antibacterial properties. The utilitarian properties were examined in the germination tests on *Lepidium sativum* for three extract dilutions (0.5, 2.5, and 10%). It was found that the extracts were similar in micro- and macroelement concentrations. Water was proved to be a good solvent to extract phenolic compounds. The algal extract produced by soaking biomass did not show inhibitory effect on *Escherichia coli* and *Staphylococcus aureus*. Only the boiled extract had an inhibitory activity against *E. coli*. Germination tests revealed a positive influence of the bioproducts on the cultivated plants. In the group treated with 10% EB, plants were 13% longer than in the control group; the content of elements B, Mo, Zn, and Na in the group treated with 10% ES was higher by 76%, 48%, 31%, and 59% than in the control group, respectively; the content of chlorophyll was 2.5 times higher in 0.5% ES than in the control group. Extracts showed the slight impact on the morphology of plants.

1. Introduction

Marine algae are considered to be one of the most important sustainable resources [1] with industrial potential [2]. Composition of macroalgae provides an excellent opportunity to study a diversity of rare biologically active compounds [3, 4] that show an array of physiological and biochemical characteristics [4, 5]. Extracts derived from algae contain such components as polysaccharides (e.g., galactan, fucoidan, alginate, and laminarin), proteins (e.g., lectins), polyunsaturated fatty acids (PUFAs), pigments (e.g., chlorophylls, carotenoids, and phycobiliproteins), polyphenols (e.g., phenolic acids, flavonoids, cinnamic acid, isoflavones, benzoic acid, and lignans, quercetin), minerals (e.g., K, Mg, Ca, and Na), and plant growth hormones (e.g., cytokinins, auxins, gibberellins, and abscisic acid) [6]. Scientific research has proven that some algal metabolites show potential antioxidant, antiproliferative [7], antidiabetic [8], antitumor [9], anti-inflammatory [10], antiallergic [11], and anti-HIV properties [12]. Because of their composition and the functional properties they are used as human food [13, 14], especially in Asia (China, Japan, and Korea) and as animal feed [15–17]. Due to their growth-stimulating activities algal formulations are used as biostimulant in crop production [15, 18, 19]. Seaweed liquid extracts have become more significant in agriculture as foliar sprays because they contain promoting hormones or trace elements (Fe, Cu, Zn, and Mn) which, added to the soil or applied to seeds, stimulate plant growth [15]. Bioproducts used in agriculture and horticulture are mainly prepared from brown seaweeds of *Ascophyllum nodosum, Ecklonia maxima,* and *Macrocystis pyrifera* [20].

The seaweed biomass collected from the Baltic Sea could be the raw material for the production of algal extracts [21]. Extraction is the most important and starting step in isolating different types of components. In the case of seaweed the extraction efficiency is reduced due to the presence of complex cell wall, which could be affected by solvent composition, temperature, time, and pH [22]. Different extraction techniques have been used to maximize biologically active compounds isolation from plant material [23], for example,

microwave assisted extraction [24, 25], supercritical fluid extraction with carbon dioxide as a solvent, Soxhlet extraction [26], enzyme-assisted extraction [27], and ultrasound-assisted extraction [28]. To this purpose different solvents can be used like ethanol, acetone, methanol-toluene [29], methanol, petroleum ether, ethyl acetate, dichloromethane, and butanol [30]. These methods require the use of expensive and toxic solvents.

To overcome limitations of conventional extraction methods and to produce algal extract we chose boiling and soaking extraction methods with distilled water. These processes are environment friendly and they do not require organic solvents. The algal extracts that we received were characterized in terms of the following:

Polyphenols.

Lipids (n-3 and n-6 fatty acids).

Microelements (B, Co, Cu, Fe, Mn, Mo, Ni, Si, and Zn).

Macroelements (Ca, K, Mg, Na, P, and S).

Antibacterial properties (gram-negative: *Escherichia coli* and gram-positive: *Staphylococcus aureus*).

We conducted germination tests on *Lepidium sativum* to examine the utilitarian properties and we determined the content of nutrients and chlorophyll in the cultivated plants and their morphology (using Scanning Electron Microscopy). The algal extract with a high content of biologically active compounds may find its future application in various industries.

2. Materials and Methods

2.1. Chemicals. All the reagents used in the experiment were of an analytical grade. Sodium carbonate, ethanol, methanol, and nitric acid were purchased from POCH SA (Poland). Folin-Ciocalteu's phenol reagent, gallic acid, and nitric acid 69% m/m, spectrally pure (Suprapur), were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Collection of Algae. The seaweeds (*Polysiphonia, Ulva,* and *Cladophora*) were collected from the Baltic Sea (Sopot, Poland), in August 2013. Algae floating freely in the coastal zone were collected from the water to minimize the contamination of raw biomass. Subsequently, the algal material was rinsed with water in order to purify it from salt and sand. Next the larger impurities (e.g., sea shells and pieces of wood) were separated; then the biomass was dried to 15% of moisture, and finally it was ground to particle size <0.3 mm [31].

2.3. Extract Production. We applied two extraction methods according to the modified procedures described by Pise and Sabale [32]. Seaweed extracts were prepared in such a way that we added 50 g of dried and shredded biomass to 150 mL of distilled water (250 mL flask). The solution was boiled in water bath for 30 minutes. In the second method we added 150 mL of distilled water to 50 g of prepared algal biomass and left it for 2 days. Afterwards each sample was centrifuged at 4250 rpm for 5 minutes and filtered with Whatman number 1 filter paper. The supernatant that we acquired was taken as a 100% algal liquid extract. For germination tests algal liquid extracts were prepared with different doses of 0.5, 2.5, and 10%. The effect of the produced extracts on the weight and height as well as chemical composition of *Lepidium sativum* was tested. We marked the extract obtained by boiling extraction as EB and by soaking extraction as ES.

2.4. Characteristics of Algal Extract. The methodology was based on the procedures described by Michalak et al. [25].

2.4.1. Multielemental Composition of Algal Extracts. Firstly, the samples of the algal biomass and cultivated plants (0.5 g) were purified from organic matter with nitric acid (5 mL) in Teflon bombs in a microwave oven (Milestone Start D, USA). Secondly, samples were diluted with redemineralized water (Millipore Simplicity) to 50 g. The samples were analyzed in three repetitions (presented as arithmetic mean, the relative standard deviation was <5%). Finally, we determined the content of elements in algal extracts, samples of algal biomass, and cultivated plants by ICP-OES (iCAP 6500 Duo, Thermo Scientific, USA) [25].

2.4.2. Phenolic Compounds in the Algal Extracts. The phenolic compounds concentrations in algal extracts expressed as gallic acid equivalents were determined with the Folin-Ciocalteu reagent [25].

2.4.3. Antibacterial Assay. Antibacterial activity (*Escherichia coli* and *Staphylococcus aureus*) was determined by the Kirby Bauer disk diffusion method, and it was recorded by measuring the diameter of the zone of inhibition (gentamicin was used as a the reference antibiotic) [25].

2.5. Utilitarian Properties of Algal Extracts

2.5.1. Germination Tests: Petri Dish Tests. To evaluate useful properties of the algal extracts, we performed the germination tests (three replicates on Petri dishes (8.9 cm), 50 seeds each) with garden cress (*Lepidium sativum*). Experiments were conducted in standardized conditions on the Jacobsen apparatus. Then each dish was treated with appropriate algal extract (5 mL). The control group (C) was treated with distilled water (5 mL). After three days, all dishes were treated with the same subsequent doses of extract/water. The tests were performed for 7 days, after which we weighed the plants and measured the height of shoot length [25].

2.5.2. Chlorophyll Content in the Cultivated Plants. To determine plant pigments, we subjected the aerial parts of cultivated garden cress to a 30-minute methanolic extraction process. The resultant colored solution was analyzed by UV-Vis spectrophotometer (Varian Cary 50 Conc. Instrument, Victoria, Australia). Measurements were made at wavelengths of $\lambda = 663$ and 645 nm. The concentration of total chlorophyll (Total Chl), chlorophyll *a* (Chl(*a*)), and

chlorophyll b (Chl(b)) was determined from the equations [33]:

$$\begin{aligned} \text{Total Chl} &= 8.02 \cdot A(663) + 20.2 \cdot A(645), \\ \text{Chl}(a) &= 12.7 \cdot A(663) - 2.69 \cdot A(645), \\ \text{Chl}(b) &= 22.9 \cdot A(645) - 4.68 \cdot A(663). \end{aligned} \quad (1)$$

2.5.3. SEM Analysis of Cultivated Plants. Stalk and leaf (the internal and external part) of *Lepidium sativum* were examined by Scanning Electron Microscopy at Wrocław University of Environmental and Life Sciences (Electron Microscope Laboratory). The samples were examined with a Scanning Electron Microscope-EVOLS 15 Zeiss (Oberkochen, Germany) operating at 20 kV. For the test, SE1 detector was used [25].

2.6. Statistical Analysis. The results were elaborated statistically by *Statistica ver. 10* (significantly different when $p < 0.05$). Distribution normality of the experimental results was assessed by the Shapiro-Wilk test whereas group differences were investigated by means of the Tukey test.

3. Results and Discussion

3.1. Characteristics of Algal Extract

3.1.1. Multielemental Composition of Algal Extracts. Table 1 presents the multielemental composition of raw algal biomass and extracts obtained by boiling and soaking with water. Generally, the extracts were similar in terms of the elemental composition. EB was especially rich in P, S, and B; on the other hand the ES contained a great amount of Ca, Mg, and Fe. It should be noted that toxic elements were extracted from the raw algal biomass in low amounts.

The multielemental composition of algal extracts resulting from different extraction methods has been known for some time (Table 1). Selvam and Sivakumar [34] presented the composition of *Ulva reticulata* extract (obtained by adding 500 g of powdered seaweeds to 5 L of water and boiling for 45 min at 60°C in a plugged conical flask). This extract was richer in ions such as Cu and Zn than extracts presented in this study (EB and ES), but Ca, K, Mg, and Na concentrations were much lower as compared to those of the Baltic extract. The Fe ions concentration (5.22 mg/L) reported in their work was higher than in EB (2.53 mg/L) but lower than in ES (17.6 mg/L). Sivasangari et al. [35] examined the mineral composition of two extracts obtained by boiling the seaweeds *Sargassum wightii* and *Ulva lactuca*. As it was shown, the macroelements content such as K, Mg, and Na was much lower than in that of Baltic extracts, but Fe ions concentration was higher. These extracts contained more microelement ions, for example, Co, Cu, and Zn, than extract EB and ES. The differences may be attributed to the mineral composition of the raw biomass and the extraction methods used. Michalak et al. [25] present the extracts obtained by microwave assisted extraction (MAE) in different temperatures (25, 40, and 60°C). It can be seen that at a lower extraction temperature, there was a lower elements concentration in the final extract. ES contained higher levels of macroelements but simultaneously contained the smallest amount of phosphorus. Microelements in all presented products were extracted at a similar level. ES contained the highest amount of Fe ions.

3.1.2. Polyphenols in Algal Extracts. Polyphenols constitute a heterogeneous group of molecules which provide a wide range of potential biological activity [36]. This class of compounds includes phenolic acids, lignins, flavonoids, tocopherols, and tannins. The use of natural antioxidants and antimicrobials can reduce the application of synthetic forms such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [37, 38]. Scientific research shows that polyphenols are good antioxidants and are effective in preventing cardiovascular and inflammatory diseases and also can be used as cancer chemopreventing agents [39]. In addition to the polyphenols from terrestrial plants (derived from gallic and ellagic acid), seaweeds have been shown as a rich source of different types of polyphenols (derived from phloroglucinol units) with unique structural properties [36, 37, 39–41]. For example, *Halimeda* (*Chlorophyceae*) contains high concentrations of polyphenols such as catechin, epicatechin, epigallocatechin gallate, and gallic acid [40]. The highest contents of these compounds are found in brown seaweeds [36].

We found out that the EB extract contained higher concentrations of polyphenols (215 mg/L) than the ES extract (173 mg/L). Water is regarded as a good solvent for isolation of phenolic compounds. López et al. [41] prepared seaweed extract by mixing (with a magnetic stirrer) dried algal powder (brown alga *Stypocaulon scoparium*) with solvents: water, water/methanol (1/1), methanol, and ethanol. Then the extracts were examined for the total phenolic content (TPC) using the Folin-Ciocalteu method. The highest amount of polyphenols was obtained for water extract (329 mg/100 g d.w. (dry weight)) and the lowest for ethanol extract (2.36 mg/100 g d.w.). Results showed a significant association between the antioxidant properties and TPC. The aqueous extract demonstrated the highest antioxidant activity and highest phenolic content. These results overlap with those reported by other researchers. Tierney et al. [42] observed that water used as the solvent for the extraction of phenolic compounds from Irish macroalgae (*Ascophyllum nodosum*, *Pelvetia canaliculata*, *Fucus spiralis,* and *Ulva intestinalis*) resulted in the highest extraction yields when compared with other solvents, for example, acetone/water (80 : 20) and ethanol/water (80 : 20). This reflects the hydrophilic nature of the majority of components found within macroalgal cells [42].

3.1.3. Antibacterial Properties of Algal Extracts. Seaweeds contain large amounts of structurally diverse secondary metabolites which offer defense against pathogens, herbivores, and decaying organisms. Compounds that exhibit the bactericidal or bacteriostatic properties include amino acids, terpenoids, phlorotannins, acrylic acid, steroids, halogenated ketones and alkanes, cyclic polysulphides, fatty acids [43],

Table 1: Multielemental composition of raw algal biomass and extracts obtained by boiling and soaking in water.

Element	Baltic seaweeds (mg/kg d.m.) ($N = 3$)	EB ($N = 3$)	ES ($N = 3$)	Extract obtained by boiling (45 min, 60°C) *Ulva reticulata* [34]	Extract obtained by boiling *Ulva lactuca* [35]	Extract obtained by MAE 25°C	Extract obtained by MAE 40°C [25]	Extract obtained by MAE 60°C
				mg/L				
Macroelements								
Ca	40292 ± 8058	333 ± 50	410 ± 61	158	—	354 ± 53	363 ± 54	365 ± 54
K	5082 ± 1016	969 ± 145	978 ± 147	175	0.980	868 ± 130	901 ± 135	951 ± 142
Mg	3181 ± 636	300 ± 45	357 ± 54	108	9.28	303 ± 45	311 ± 46	322 ± 48
Na	6354 ± 1271	1239 ± 248	1302 ± 260	295	5.04	1050 ± 211	1200 ± 240	1250 ± 250
P	1155 ± 231	34.7 ± 5.2	5.22 ± 0.78	—	—	9.52 ± 1.43	18.3 ± 2.7	32.9 ± 4.9
S	8614 ± 1723	670 ± 100	599 ± 90	—	—	562 ± 84	582 ± 87	702 ± 105
Microelements								
B	97.8 ± 14.7	6.50 ± 0.97	2.62 ± 0.39	—	—	3.30 ± 0.5	3.44 ± 0.51	4.74 ± 0.71
Co	2.84 ± 0.43	0.0100 ± 0.0025	0.0100 ± 0.0025	—	0.201	0.012 ± 0.003	0.012 ± 0.003	0.0135 ± 0.0034
Cu	12.7 ± 1.9	0.140 ± 0.021	0.0200 ± 0.005	1.15	3.02	0.117 ± 0.0117	0.148 ± 0.022	0.108 ± 0.016
Fe	6660 ± 1332	2.53 ± 0.38	17.6 ± 2.6	5.22	0.417	1.19 ± 0.2	2.17 ± 0.3	4.47 ± 0.70
Mn	232 ± 35	2.43 ± 0.36	3.71 ± 0.56	—	0.050	2.52 ± 0.37	2.62 ± 0.39	3.07 ± 0.46
Mo	0.310 ± 0.046	0.0200 ± 0.005	0.000	—	—	0.001 ± 0.000	0.179 ± 0.026	0.0108 ± 0.0027
Ni	5.23 ± 0.78	0.130 ± 0.019	0.120 ± 0.018	—	—	0.108 ± 0.016	0.111 ± 0.016	0.132 ± 0.019
Si	907 ± 136	9.10 ± 1.36	9.73 ± 1.46	—	—	3.10 ± 0.46	6.69 ± 1	11.9 ± 1.8
Zn	64.9 ± 9.7	0.240 ± 0.036	0.100 ± 0.015	1.25	1.02	0.0746 ± 0.0112	0.201 ± 0.03	0.169 ± 0.025
Toxic metals								
As	3.90 ± 0.507	0.170 ± 0.022	0.160 ± 0.021	—	—	0.246 ± 0.032	0.150 ± 0.019	0.198 ± 0.025
Cd	0.710 ± 0.092	<LLD	<LLD	—	—	<LLD	0.001 ± 0.000	0.001 ± 0.000
Pb	7.03 ± 0.91	0.0400 ± 0.008	0.0100 ± 0.0025	—	—	0.0098 ± 0.002	0.0104 ± 0.0021	0.032 ± 0.006

<LLD: below limit of detection.

proteins, polyphenols, polysaccharides, and pigments (e.g., chlorophyll and carotenoids) [22].

We examined the antibacterial activity of the extracts against gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria. ES showed no inhibitory activities, whereas EB revealed an inhibitory activity (18 mm) against *E. coli* but did not have the zone of inhibition against *S. aureus*. The control group (gentamicin) showed the inhibitory zone (32 mm) against *E. coli* and *S. aureus* (26 mm). The literature data regarding the antibacterial properties of aqueous algal extracts are divergent. Mohana Priya and Ali [44] presented that the aqueous extract of *Ulva fasciata* showed the antibacterial activity against *E. coli* (16 mm) and *S. aureus* (15 mm). Christobel et al. [45] presented that 100% *Ulva fasciata* aqueous extract had inhibitory activity against *S. aureus* (10 mm) and *E. coli* (9 mm). Mansuya et al. [46] reported that aqueous extracts of *Ulva reticulata* did not inhibit *E. coli* growth, but *Cladophora glomerata* and *Ulva lactuca* extracts showed inhibition. Alghazeer et al. [47] wrote that aqueous extracts of *Ulva lactuca, Enteromorpha compressa, Enteromorpha prolifera,* and *Enteromorpha* spp. demonstrated the antimicrobial activity against *S. aureus* (11-12 mm) and *E. coli* (11-12 mm). Selvi et al. [48] observed that aqueous extracts of *Enteromorpha compressa, E. intestinalis, Ulva lactuca,* and *U. fasciata* showed trace antibacterial activity for both strains: *S. aureus* and *E. coli*. The differences in antibacterial properties of algal extracts may be caused by the composition of seaweeds, place, season of their collection, extraction techniques used, solvents (its polarity), and parameters of the extraction process [22, 49].

3.2. Utilitarian Properties of Algal Extracts. Seaweeds, due to their content of organic matter and fertilizer nutrients, have been used as soil conditioners for centuries [18]. The first practical method for liquefying seaweed for agricultural use was developed in 1949 [50]. Seaweed extract can produce beneficial effect on plants, such as early seed germination, improved crop yield, elevated resistance to abiotic and biotic stress, and also enhanced postharvest shelf-life of perishable products [18]. Great advantages of seaweed products, compared with conventional crop protection products, are that they are biodegradable, do not show toxicity, and exhibit activity in low doses (<15 L/ha) [50, 51]. In the present study we investigated the effect of algal extracts on total height, dry weight, content of chlorophyll and nutrients, and morphology of garden cress (*Lepidium sativum*). For the germination experiments, we prepared dilutions (0.5, 2.5, and 10%) of the raw extract.

3.2.1. Total Height of the Cultivated Garden Cress. For both extracts, plant height ($N = 20$ from each group, from each replicate) was determined for all three dilutions (0.5, 2.5, and 10%). The extracts under study exhibited varying degrees of stimulatory effect on plant growth. Table 2 presents the results. In most cases, plants in the experimental groups were higher than those in the control group, treated with distilled water. The best results were achieved for the plants treated with 10% EB; these were 13% longer than those in the control group. The lowest plants were observed in groups treated with 0.5% EB (by ~3.0% shorter than plants in the control group) and 2.5% EB (by ~5.0% shorter than control). The differences were statistically significant (for $p < 0.05$). Latique et al. [52] demonstrated that the application of extracts (25 and 50%), resulting from boiling the fresh biomass (*Ulva rigida*) in a distilled water, provided significant effects on bean growth (*Phaseolus vulgaris* L.). The best effect was noted in the group treated with a 25% dilution. Similar results were reported by Gireesh et al. [53], who tested extracts produced by boiling green alga *Ulva lactuca* in distilled water for an hour. Subsequently, the series of 5, 10, 20, 30, 40, and 50% concentrations were prepared and tested on seedling growth of *Vigna unguiculata* L. Walp, shoot and root length. The best results were obtained for the 20% aqueous seaweed extract. Higher concentrations (≥40%) yielded inhibited germination [53]. Kavipriya et al. [54] investigated the effect of extracts from different seaweeds including *Ulva lactuca*, produced by autoclaving the biomass with distilled water (121°C, 30 min), on *Vigna radiata* (green gram) seed germination and growth parameters. Seaweed liquid extracts were prepared with 0.1, 0.2, 0.3, 0.4, and 0.5% doses. The best results were obtained for 0.2% dilution: plants were 1.8 times higher than in the control. Pise and Sabale [32] investigated the effect of three seaweeds including *U. fasciata*. Different methods, using algal powder, were used for the preparation of extracts. In one of them, dried biomass was boiled for an hour in distilled water. In another, algae were soaked in distilled water for two days. The efficiency of the preparations (10, 25, and 50%) was examined on *Trigonella foenum-graecum* L. Shoot growth was increased by all the extracts and the maximum value was recorded for the 50% concentration.

Table 2: The results of total height of the cultivated garden cress in the examined groups ($N = 3$).

Group	Average height of cress (cm) ± SD*
C	5.36 ± 0.59^{a}
EB 0.5%	5.21 ± 0.59^{bcde}
EB 2.5%	5.10 ± 0.71^{fghi}
EB 10%	6.07 ± 0.61^{abf}
ES 0.5%	5.93 ± 0.75^{cg}
ES 2.5%	5.78 ± 0.93^{dh}
ES 10%	5.90 ± 0.86^{ei}

*Three replicates for each group; 20 randomly selected plants in each replicate were measured.
a, b, c, d, . . .: statistically significant differences for $p < 0.05$.

3.2.2. Weight of the Cultivated Plants. We found out that dry mass of the cultivated garden cress, taking into account both methods of the extraction and the dilutions of the extracts, was comparable in all the groups (Table 3). We observed no influence of algal extract concentration on *Lepidium sativum* dry weight. Gireesh et al. [53] showed that the 20% concentration of *Ulva lactuca* aqueous extract increased *Vigna unguiculata* L. Walp dry weight (plants were ~9% higher than in the control group). It was observed that higher

Table 3: The dry weight of garden cress in various experimental groups ($N = 3$).

Group	Average dry weight of cress (g) ± SD*
C	0.0739 ± 0.0033
EB 0.5%	0.0727 ± 0.0037
EB 2.5%	0.0670 ± 0.0016
EB 10%	0.0733 ± 0.0033
ES 0.5%	0.0712 ± 0.0022
ES 2.5%	0.0680 ± 0.0010
ES 10%	0.0703 ± 0.0014

*Three replicates for each group; all plants in each replicate were weighed.

concentrations (30, 40, and 50%) reduced the dry weight of plants. Kavipriya et al. [54] showed that the best results of extract (range of concentrations 0.1–0.5%) obtained from *Ulva lactuca*, in promoting the dry weight of plants, were in the group treated with 0.2% concentration (24% higher than in control group). In the Pise and Sabale [32] study, dry weight of plants was the highest when 50% concentration of *Ulva* extracts obtained by boiling and soaking methods was applied.

3.2.3. Multielemental Composition of the Cultivated Garden Cress. The application of seaweed extracts can increase the content of micro- and macroelements in the cultivated plants [25, 55]. In the present study we observed that the highest content of micro- and macroelements in *Lepidium sativum* occurred in the groups treated with 0.5% EB, as well as 0.5% and 10% ES (Table 4). Among these three extracts, the best results were obtained for 10% ES. The content of B (76%), Cu (2.6 times), Mn (20%), Mo (48%), Ni (2.4 times), Zn (31%), K (15%), Mg (7%), Na (59%), and S (4%) was higher than in the control group. Michalak and Chojnacka [55] presented the results that showed that the cultivated garden cress contained mainly these micro- and macroelements, which occurred at the largest concentrations in the algal extract. Plants treated with 100% extract were rich in macroelements such as K (3 times more as compared to the control group treated with water), S (44% more), and Ca (about 35% more). Among the trace elements, the largest quantities in biomass were observed for B (5% more than in control) and Mn (2 times more).

3.2.4. Chlorophyll Content in the Cultivated Cress. The concentrations of chlorophyll in cultivated plants are presented in Table 5. In most cases, total chlorophyll concentration in *Lepidium sativum* in experimental groups was higher than in the control group. The highest content of total chlorophyll in plant was in the group treated with 0.5% ES (2.5 time more than in the control group). It can be seen that, with increasing concentration of ES, the content of chlorophyll in plants decreased. For EB, the best results were obtained for the 2.5% concentration. A high content of elements such as Mg, Fe, and Cu in Baltic algae and consequently in extracts [25] and also the presence of betaine, which causes the increase of the concentration of chlorophyll in leaves [56], are related to the stimulatory effect on chlorophyll synthesis. Results of this study showed that the examined algal extracts increased plant productivity, resulting in increased chlorophyll content. Gireesh et al. [53] reported that lower concentrations of the aqueous *Ulva lactuca* extract have promoted the chlorophyll content of *Vigna unguiculata* (even about 20%). It was also noticed that the higher concentrations (>20%) decreased the chlorophyll content in plants. Gaikwad et al. [57] observed that foliar application of 0.1% aqueous extract of *Ulva lactuca* L. enhanced chlorophyll content in *Solanum melongena* L. when compared with control group. Osman and Salem [58] showed that the aquatic extracts (0.4 and 0.6%) obtained from *Ulva lactuca* significantly increased the content of chlorophyll *a* and *b* in sunflowers (*Helianthus annuus* L.). Pise and Sabale [32] observed that extracts obtained from *Ulva* increased the content of photosynthetic pigments in harvested *Trigonella foenum-graecum* L.

3.2.5. SEM Analysis of Cultivated Plants. In the present paper, to evaluate the effect of EB and ES extracts on *Lepidium sativum* morphology, stalk, and leaf (the internal and external part) we used Scanning Electron Microscopy. Figure 1 presents observations for two magnifications (500 and 2000). The morphological studies showed significant stem changes treated with both aqueous extracts. In the group treated with EB, we observed the skin with clearly setting parallel fibers of the surface layer. In the case of garden cress treated with ES it can be noticed that the surface layer of the stalk (Figure 1(a)) was significantly shrunken. SEM showed also a considerable morphological changes of the lower epidermis of the plant leaves treated with both extracts. In both groups we observed the shrinking of the cuticles and the stoma (Figure 1(b)). This study showed slight changes in an external part of epidermal leaf and the enlargement of the stomas (Figure 1(c)). Morphological studies showed the impacts of the extracts on garden cress morphology, mainly in stoma composition and size.

4. Conclusions

Considering the above findings, the seaweed extracts derived from *Polysiphonia, Ulva*, and *Cladophora* could be used as nontoxic biostimulants of plant growth. It was observed that obtained extracts were similar in terms of the concentration of the microelements and macroelements and low content of toxic elements. This research shows that water is a good solvent to extract phenolic compounds. In most cases, algal extracts did not show antibacterial activity against *E. coli* and *S. aureus*. Only the EB extract presented an inhibitory activity against *E. coli*. Germination tests showed a positive influence of obtained products on height, multielemental composition, and the content of chlorophyll in the cultivated plants (*Lepidium sativum*) and also showed the impacts on morphology of garden cress. The best product which increased the total height of garden cress was 10% EB. Plants in this group were 13% longer than in the control group. The extract which decreased plant length by 3% was 0.5% EB. The dry mass of the cultivated garden cress was comparable

Table 4: Multielemental composition of the cultivated garden cress (mg/kg d.m., $N = 3$).

Element	C	EB			ES		
		0.5%	2.5%	10%	0.5%	2.5%	10%
Macroelements							
Ca	11161 ± 394	12752 ± 279	13126 ± 693	12956 ± 902	10818 ± 508	11881 ± 288	10475 ± 644
K	58067 ± 3802	65020 ± 291	67021 ± 1547	67774 ± 1430	68562 ± 190	67774 ± 2705	66983 ± 623
Mg	6974 ± 68	7188 ± 419	7210 ± 2	6854 ± 340	7662 ± 307	7409 ± 35	7456 ± 53
Na	1850 ± 8	1848 ± 181	2140 ± 24	3132.50 ± 92.79	2152.53 ± 97.27	2236.00 ± 170.10	2933.15 ± 51.10
P	16475 ± 964	16626 ± 798	16236 ± 301	14727 ± 686	16390 ± 170	17197 ± 211	16550 ± 83
S	15508 ± 939	15139 ± 488	14921 ± 166	12786 ± 999	15193 ± 260	17572 ± 1009	16155 ± 236
Microelements							
B	7.30 ± 0.82	11.3 ± 0.2	12.7 ± 0.52	12.7 ± 3.1	20.8 ± 13.0	15.1 ± 3.0	12.8 ± 0.9
Co	0.08 ± 0.03	0.11	0.09 ± 0.03	0.04 ± 0.01	0.12 ± 0.12	0.12 ± 0.05	0.21 ± 0.13
Cu	4.24 ± 0.07	5.75 ± 0.65	5.43 ± 0.85	4.72 ± 0.04	6.25 ± 1.21	9.80 ± 5.46	11.19 ± 2.49
Fe	278 ± 11	288 ± 46	279 ± 33	263 ± 13	198.79 ± 6.07	207 ± 3.0	227.77 ± 0.96
Mn	41.9 ± 1.85	47.6 ± 3.2	46.1 ± 1.6	45.0 ± 2.3	49.09 ± 0.13	49.04 ± 0.62	50.39 ± 1.00
Mo	1.20 ± 0.01	2.17 ± 0.47	1.73 ± 0.07	1.89 ± 0.14	1.79 ± 0.16	1.63 ± 0.14	1.77 ± 0.09
Ni	0.63 ± 0.16	1.44 ± 0.54	1.10 ± 0.02	0.84 ± 0.23	1.26 ± 0.13	1.23 ± 0.63	1.51 ± 0.13
Si	182 ± 22	271 ± 35	244 ± 38	226 ± 8.0	245 ± 71.49	192.25 ± 20.15	180.45 ± 8.41
Zn	58.22 ± 0.71	81.9 ± 2.7	88.2 ± 4.1	76.8 ± 0.2	88.6 ± 2.18	83.16 ± 0.04	76.35 ± 0.24
Toxic metals							
As	0.35 ± 0.12	0.17 ± 0.07	0.0521 ± 0.0104	0.36 ± 0.05	0.49 ± 0.59	0.15 ± 0.10	<LLD
Cd	0.26 ± 0.04	0.28 ± 0.01	0.24 ± 0.01	0.21 ± 0.01	0.31 ± 0.02	0.30 ± 0.02	0.24 ± 0.03
Pb	1.19 ± 0.01	1.52 ± 0.39	1.48 ± 0.05	1.53 ± 0.46	1.92 ± 0.62	1.44 ± 0.09	1.28 ± 0.01

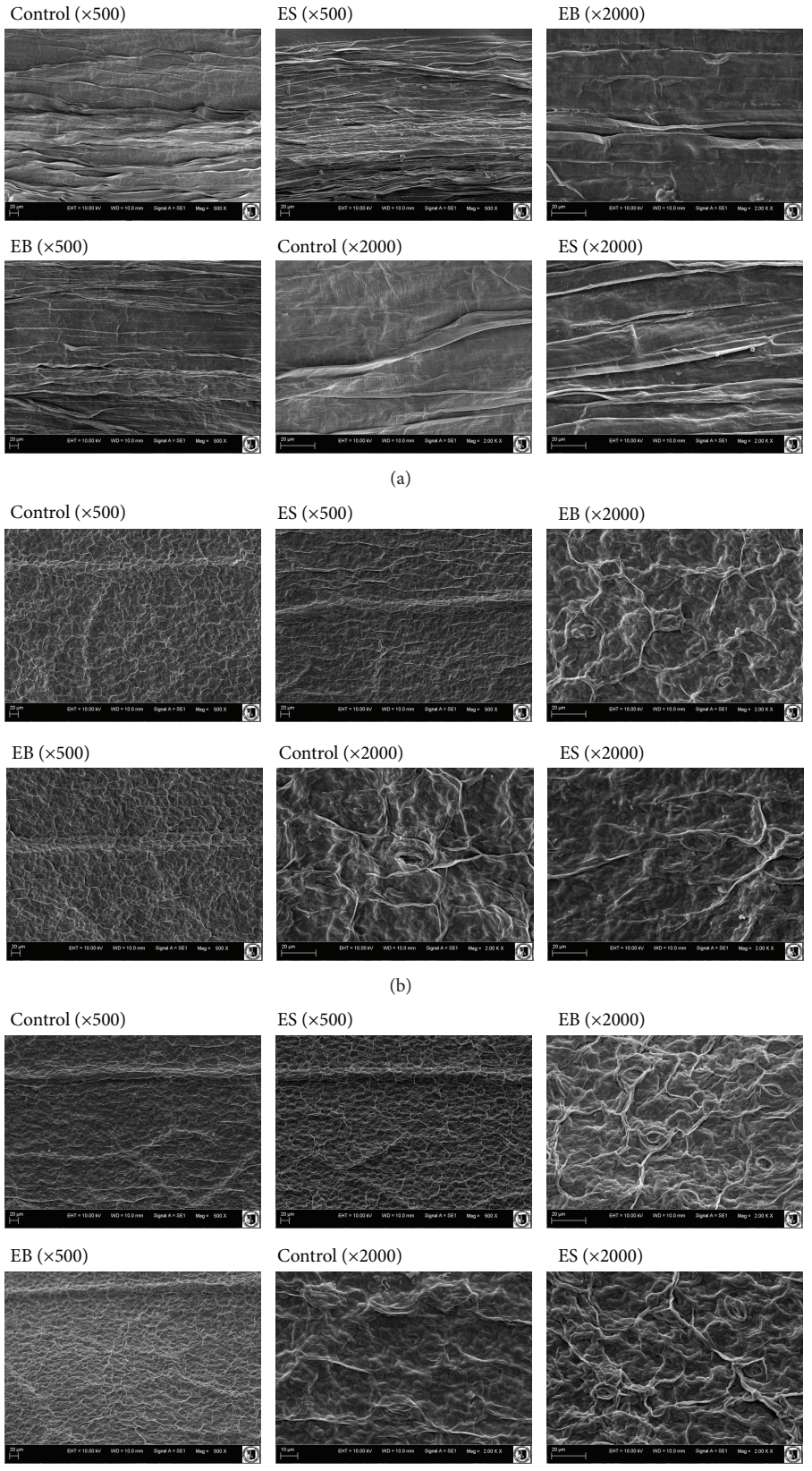

Figure 1: The influence of algal extracts produced by boiling and soaking methods on plant morphology: (a) stalk, (b) leaf, internal part, and (c) leaf, external part of plant.

Table 5: Chlorophyll concentration in the cultivated garden cress (mg/L).

Sample	Concentration of chlorophyll *a*	Concentration of chlorophyll *b*	Total chlorophyll concentration
C	15.5	5.95	21.4
EB 0.5%	23.9	9.00	32.9
EB 2.5%	26.4	9.55	36.0
EB 10%	14.9	5.97	20.8
ES 0.5%	12.2	42.2	54.4
ES 2.5%	10.0	34.9	44.9
ES 10%	9.4	32.7	42.1

in all groups. Mainly 10% ES influenced content of micro- and macroelements in *Lepidium sativum*. In the group treated with 0.5% ES we observed the highest content of total chlorophyll in plant. Used seaweed species can be considered as a potential source of nutrients for plants and be used in agriculture and horticulture to attain better germination, growth, and yield. Because of the reported multifunctional properties of seaweed extracts, their exploitation as a source of biological active compounds could be possible.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Biotransformation of Cholesterol and 16α,17α-Epoxypregnenolone and Isolation of Hydroxylase in *Burkholderia cepacia* SE-1

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The metabolism of cholesterol is critical in eukaryotes as a precursor for vitamins, steroid hormones, and bile acids. Some steroid compounds can be transformed into precursors of steroid medicine by some microorganisms. In this study, the biotransformation products of cholesterol and 16α,17α-epoxypregnenolone produced by *Burkholderia cepacia* SE-1 were investigated, and a correlative enzyme, hydroxylase, was also studied. The biotransformation products, 7β-hydroxycholesterol, 7-oxocholesterol, and 20-droxyl-16α,17α-epoxypregn-1,4-dien-3-one, were purified by silica gel and Sephadex LH-20 column chromatography and identified by nuclear magnetic resonance and mass spectroscopy. The hydroxylase was isolated from the bacterium and the partial sequences of the hydroxylase, which belong to the catalases/peroxidase family, were analyzed using MS/MS analyses. The enzyme showed activity toward cholesterol and had a specific activity of 37.2 U/mg of protein at 30°C and pH 7.0.

1. Introduction

Cholesterol has an important role in chemistry, medicine, and biology and is an essential structural component of animal cell membranes [1]. The metabolic products of cholesterol are the precursors of vitamins, steroid hormones, and bile acids [2, 3]. Since Murray and Peterson first discovered that *Rhizopus nigricans* could transform progesterone into 11α-hydroxyl progesterone, it has attracted considerable attention [4]. Low-cost natural steroids, such as cholesterol, can be used as starting materials for synthesizing many bioactive steroids [5]. Therefore, an understanding of the bacterial metabolism of cholesterol could be useful in the development of biotechnological tools for the transformation of steroid components via metabolic engineering. Many different microorganisms can use environmental cholesterol and related sterols as a common carbon source. The microbial transformation of steroids has great advantages over chemical processing methods, especially because it is very difficult to process those steroids using chemical methods. Recently, various mechanisms and metabolic pathways for steroids in microbes have been studied. Some bacteria and fungi can hydroxylate at different locations on the steroid molecule [6–8]. Such reactions could be developed into an important technique; particularly, the ability to perform 7-hydroxylation of steroid compounds has potential use. For example, *Acremonium stricture* transformed 4-pregnene-3,20-dione into 7β,15α-dihydroxyl-4-pregnene-3,20-dione [9]. The filamentous fungus, *Aspergillus fumigatus*, efficiently hydroxylated exogenous progesterone, producing 11α- and 15β-hydroxyprogesterone as major products and 7β-hydroxyprogesterone as a minor product [10]. Bacillus strains transformed 3β-hydroxyl-4-pregnene-20-one into 3β,7α-dihydroxyl-4-pregnene-20-one [11]. Some 7-hydroxyl derivatives of steroid play an important role in medicine. For example, 7-hydroxyl derivatives of dehydroepiandrosterone have a curative effect against cancer and Alzheimer's disease and show antiglucocorticoidal action and decrease fat and enhance immune function [12, 13]. The same functions are exhibited by 7-hydroxyl derivatives of 4-pregnene-3,20-dione [12]. Some studies indicate that 7β-hydroxycholesterol can inhibit cell hyperplasia and tumor cell multiplication [14, 15]. In our study, we report for the first time the transformation of cholesterol and 16α,17α-epoxypregnenolone by strain *Burkholderia cepacia*

SE-1. The transformation products, 7β-hydroxycholesterol, 7-oxocholesterol, and 20-droxyl-16α,17α-epoxypregn-1,4-dien-3-one, and a correlative enzyme, hydroxylase, were investigated.

2. Materials and Methods

2.1. Materials. Cholesterol and 16α,17α-epoxypregnenolone were purchased from Xian Blue Sky Biological Engineering Co., Ltd. (China). Silica gel GF254 plates and silica gel 200–300 mesh were obtained from Qingdao Marine Chemical Ltd. (China). NMR spectra were recorded on Bruker AV-600 MHz instruments (Bruker, Switzerland) with TMS as an internal standard. MS spectra were recorded on an HPLC-MS ZQ4000/2695 (Waters, USA). Sephadex LH-20 (Amersham Biosciences, Sweden) was used for column chromatography. Petroleum ether, acetone, and chloroform were of analytical grade and methanol for High Performance Liquid Chromatography (HPLC) was of chromatography grade (Waters Symmetry Shield RP 18, 150 mm × 3.9 mm 5 μm).

2.2. Methodology

2.2.1. Screening and Isolation of the Bacterium. Strains that metabolized cholesterol were isolated from forest field soil on the campus of Jiangxi Agricultural University, Nanchang, China. Microorganisms that could metabolize cholesterol were screened with a screening medium. The enrichment media were composed of (g/L) Peptone (Aobox, Beijing) 10, Beef Extract (Aobox, Beijing, China) 3, $MgSO_4$ 0.25, K_2HPO_4 0.25, $FeSO_4{\cdot}7H_2O$ 0.001, NaCl 0.05, and $CaCl_2$ 0.001. The screen media were composed of (g/L) $MgSO_4$ 0.25, K_2HPO_4 0.25, $FeSO_4{\cdot}7H_2O$ 0.001, NaCl 0.05, $CaCl_2$ 0.001, agar15, pH 7.0, and 1.0 g/L cholesterol as sole carbon source. Cholesterol and sodium dodecyl sulfate (SDS) were mixed together at the molar ratio of 1 : 1 for 20 min with an ultrasonic mixer to increase the solubility of cholesterol in water. The medium was solidified with 1.5% (wt. vol^{-1}) agar. The culture medium contained (g/L) Peptone (Aobox, Beijing) 10, Beef Extract (Aobox, Beijing) 3, $MgSO_4$ 0.25, K_2HPO_4 0.25, $FeSO_4{\cdot}7H_2O$ 0.001, NaCl 0.05, and $CaCl_2$ 0.001 and the microorganisms were grown at 30°C, with shaking at 220 r/min for 24 h. Uninoculated culture medium incubated under the same conditions was used as a control.

2.2.2. Identification of the Bacterium. Only one bacterial species with the desired metabolic activity was isolated and it was identified and characterized using standard biochemical tests from *Bergey's Manual of Determinative Bacteriology* [17]. Cellular morphology was observed with a transmission electron microscope (H7650, Japan). This species was identified by comparing the sequence of the 16S rDNA gene, which was isolated with a UNIQ-10 column genomic DNA kit, to the database of NCBI GenBank, genomic DNA. The DNA was sequenced by a commercial automated sequencing system provided by Sangong Biotech Ltd. (Shanghai, China). The bacterium genomic 16S rDNA was amplified by PCR. The sense (5′-AGAGTTTGATCCTG GTCAG-3′) and antisense (5′-AAGGAGGTGATCCAGCCGCA-3′) primers universal to prokaryotes were used. A phylogenetic tree was constructed using the neighbor-joining method with MEGA (5.0) software. The strain was deposited in the microbial laboratory of the College of Bioscience and Bioengineering, Jiangxi Agricultural University.

2.2.3. Assay of Biotransformation Products. A single colony of *Burkholderia cepacia* SE-1 from an agar plate of the screening medium was inoculated into 30 mL of culture medium in 100 mL flasks and incubated at 30°C, while shaking at 220 rpm for 24 h. The culture was subsequently inoculated into 300 mL of culture medium in 1000 mL flasks and incubated at 30°C and 220 rpm for 24 h. Cells were collected by centrifugation at 8000 ×g for 10 minutes at room temperature and then resuspended in 300 mL of reaction mixture containing 0.07 M Na_2HPO_4/KH_2PO_4 buffer (pH 8.0) and 1.0 g/L cholesterol or 16α,17α-epoxypregnenolone in a 1000 mL flask and incubated at 30°C and 220 rpm for 48 h. After the reaction was complete, cells were harvested from the culture by centrifugation for 10 minutes at 8000 ×g, at room temperature. Cells were sequentially extracted with 50, 30, and 20 mL chloroform, respectively. Therefore some products were solved into chloroform. The chloroform solution was collected and evaporated *in vacuo*. The chloroform solution (5 μL) of transformed products was spotted onto a 0.25 mm silica gel GF254 thin layer chromatography (TLC) plate, developed in a petroleum ether : acetone (3 : 1, v/v) solvent system and visualized by heat after spraying with 10% H_2SO_4-anhydrous ethanol.

2.2.4. Purification and Structural Characterization of the Biotransformation Products. The concentrating chloroform solution was mixed with a little silica gel and subjected to silica gel column chromatography (CC, silica gel (200–300 mesh)) and eluted with petroleum ether : acetone (95 : 5–70 : 30) by monitoring with TLC. The fraction containing the conversion products was purified by Sephadex LH-20 gel column chromatography methods and eluted with $CHCl_3$-MeOH 1 : 1. 7β-Hydroxycholesterol was tested by HPLC. The column was eluted with methanol at a flow rate of 1.0 mL/min. The elution was monitored by measurement of the absorbance at 215 nm. ESI-MS spectra of the purified compounds were acquired on an HPLC-MS ZQ4000/2695 quadrupole LC-MS in the positive and negative modes. NMR spectra were recorded in $CDCl_3$-d on a Bruker AM-600 NMR instrument (600 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR).

2.2.5. Hydroxylase Activity Was Assayed by TLC. To hydroxylase activity assay, 0.1 mL of a cholesterol solution (10 mg cholesterol dissolved in 1 mL of acetone) was added to 0.5 mL of the elute, 0.2 mM NAD^+ was added as a cofactor, and the mixture was incubated at 30°C, pH 7.0 for 1 h. Petroleum ether (0.5 mL) was added to the reaction mixture. The solution was shaken and allowed to settle and then 5 μL of the petroleum ether solution was spotted onto a 0.25 mm silica gel GF_{254} TLC plate, developed in petroleum ether : acetone (3 : 1, v/v) solvent system and visualized by heat after spraying with 10% H_2SO_4-anhydrous ethanol.

2.2.6. Purification of the Enzyme. *Burkholderia cepacia* SE-1 was grown at 30°C for 24 h in the culture medium and then the culture was centrifuged at 4,000 ×g for 10 minutes at 4°C. Cells were dissolved in a buffer solution of 10 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA, lysed ultrasonically, and centrifuged at 8,000 ×g for 30 minutes at 4°C. The supernatant was extracted with an $(NH_4)_2SO_4$ solution at 70% saturation at 4°C for 24 hours. The precipitate was recovered by centrifugation at 12,000 ×g for 30 minutes at 4°C and redissolved in a buffer solution containing 10 mM Tris-HCl, pH 8.0. This solution was dialyzed against the same buffer at 4°C until a 10 mL solution was achieved. The solution was placed on a column (2.5 × 50 cm) of DEAE-cellulose preequilibrated with 10 mM Tris-HCl, pH 8.0, buffer and washed with a linear gradient of Tris-HCl buffer solutions containing 40–120 mM NaCl, at a flow rate of 60 mL/h. The elute (20 mL) was collected in a conical flask.

The fraction of activity of cholesterol hydroxylase was going to be separated further. The fractions were freeze-dried to a 5 mL volume and loaded on a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (2.5 × 50 cm). This column had been preequilibrated with a 10 mM Tris-HCl (pH 8.0) buffer containing 50 mM NaCl and was eluted with the same buffer at a flow rate of 30 mL/h. The elute (10 mL) was collected in conical flasks. The hydroxylase activity was assayed by TLC as described above. The fractions containing cholesterol hydroxylase were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and freeze-dried to a 0.5 mL volume and then subjected to polyacrylamide gel electrophoresis. After electrophoresis, the protein bands were cut from two sides of the gel and stained with 0.025% (w/v) of Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and then destained in 50% methanol. By comparing the other section of the gel which was unstained with the two pieces of gel section stained, the protein bands of interest were located and excised and dissolved in 10 mM Tris-HC. The activity in the protein bands was assayed by TLC as described above. All of the purification steps were performed at 4°C. Protein was detected by absorbance at 280 nm.

2.2.7. The Hydroxylase Activity Was Evaluated by HPLC. The hydroxylase activity was assayed by measuring the quantity of 7β-hydroxycholesterol produced. Petroleum ether was used to extract 7β-hydroxycholesterol from the reaction mixture, which was solubilized with methanol after petroleum volatilization.

One unit of enzymatic activity was defined as the amount of protein required to hydroxylate 1 μmol of cholesterol per minute at 30°C. Reverse-phase chromatography on an ODS column (HPLC) was employed to determine 7β-hydroxycholesterol with a preparation of 7β-hydroxycholesterol (98%) as the standard. The column was eluted with methanol at a flow rate of 1.0 mL/min and the elution was monitored by measurement of the absorbance at 205 nm.

2.2.8. Amino Acid Sequence Analysis. Purified enzyme was sent to Sangong Biotech Ltd. (Shanghai, China) for MS and MS/MS analyses with matrix assisted-laser desorption ionization- (MALDI-) time of flight (TOF) and MALDI-TOF-TOF methods.

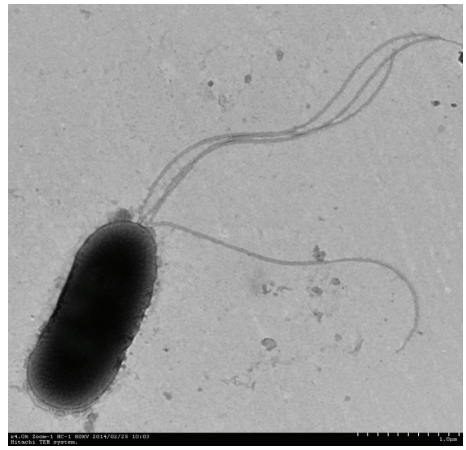


Figure 1: Scanning electron micrograph of *Burkholderia cepacia* SE-1 strain, ×32K.

Protein was digested with trypsin and then analyzed with a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). The MALDI MS parameters were designed with MS acquisition in the reflector mode, positive ion mode, mass range 850–4,000 (mass/charge (m/z)), and minimum signal/noise (S/N) set at 10 for MS acquisition. Twenty-five of the strongest precursors were chosen for MS/MS, and a minimum S/N 30 was used for MS/MS precursors. The Applied Biosystems GPS Explorer™ v3.6 program was used for a combined search of MS and MS/MS data, with Mascot as the search engine (available at http://www.matrixscience.com/). Searches allowed for carbamidomethylation and monoisotopic oxidation, 100 ppm of peptide mass or parent tolerance, and a maximum of one missed trypsin cleavage. Peptide tolerance and MS/MS tolerance were both 0.5 Da. Proteins with a statistically significant ($P < 0.05$) protein score were considered as identified with confidence (based on combined mass and mass/mass spectra). Redundancy of proteins that appeared in the database under different names and accession numbers was eliminated.

3. Results

3.1. Identification of the Microorganism. The isolated organism was rod-shaped and 0.8–1.0 μm × 1.6–3.2 μm in size, Gram-negative, with a bipolar flagellum. The bacterial colony was round, smooth, moist, and opaque, with smooth edges, and had a straw yellow color after 24 h growth at 36°C on the plate. An electron micrograph showing strain morphology is shown in Figure 1.

We analyzed a partial sequence of the 16S rDNA sequence of this strain. The 16S rDNA gene (approximately 1525 bp) from the isolated bacterial strain was sequenced and the data was submitted to GenBank, which was assigned the accession number KF681774. The amplified sequence showed a 99% similarity to the 16S rRNA genes of *Burkholderia cepacia* strain 17759 from the American Type Culture Collection

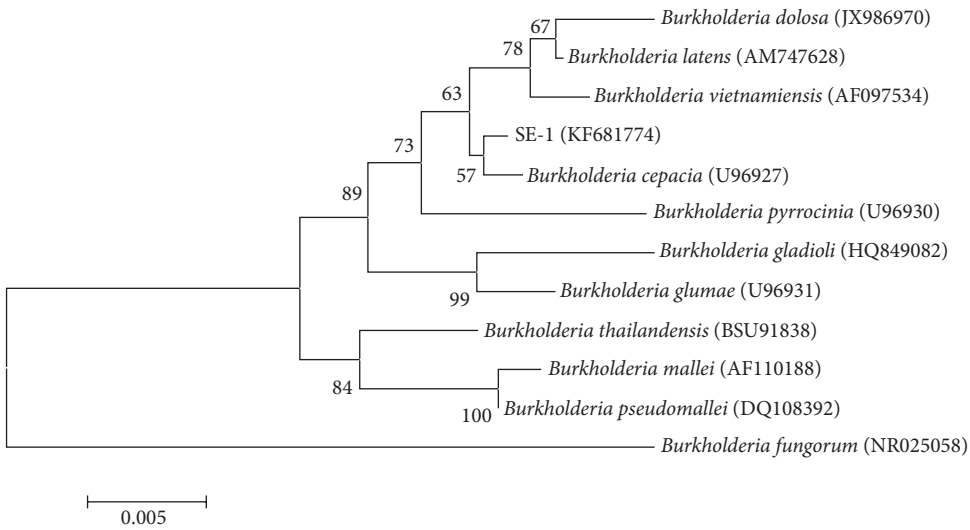


Figure 2: Phylogenetic tree based on 16S rRNA sequences of selected strains. Numbers in parentheses represent the sequences accession number in GenBank. The number at each branch point is the percentage supported by bootstrap. Bar, 0.5% sequence divergence.

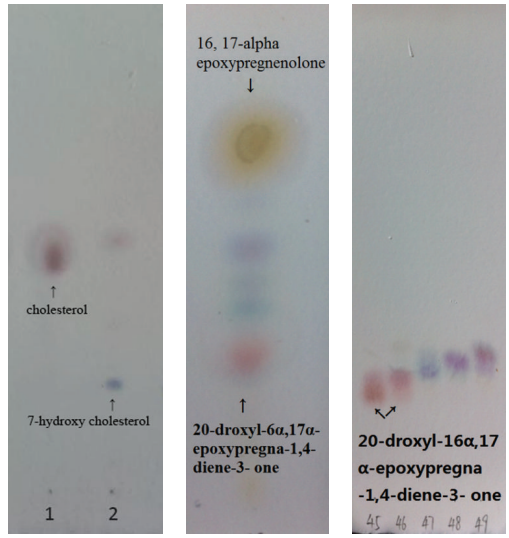
(ATCC) (GenBank accession number AY741334, results not shown) and *B. cepacia* ATCC 17762 (GenBank accession number AY741335). We built a phylogenetic tree by comparing the sequence of the 16S rDNA from our isolate with sequences from 11 standard *Burkholderia* genus stains. Based on the morphological characteristics and 16S rDNA sequence analysis, our strain was identified as *Burkholderia cepacia* SE-1. The phylogenetic tree based on the 16S rDNA sequence showed that strain SE-1 was classified as *Burkholderia* sp. (Figure 2). No one has yet reported cholesterol hydroxylase from the genus *Burkholderia*, so we studied the cholesterol hydroxylase from the isolated *Burkholderia cepacia* SE-1 strain further.

3.2. Separation and Analysis of Biotransformation Products. After the reaction was complete, cells were harvested from the culture by centrifugation for 10 minutes at 8000 ×g, at room temperature. The supernatant and cells were extracted with chloroform, respectively. The thin layer chromatography (TLC) plate showed a deep blue spot in the solution of extracted cells from strain SE-1. It revealed that the blue substance did not exist in the culture medium without inoculated strain and the supernatant. It also proved that hydroxylase enzyme is expressed intracellularly and the biotransformed products are expressed intracellularly. 7β-Hydroxycholesterol (128 mg) and 7-oxocholesterol (32 mg) were separated from the reaction mixture with a silica gel and Sephadex LH-20 gel column chromatography. Similarly, 20-droxyl-16α,17α-epoxypregn-1,4-dien-3-one was separated from the reaction mixture of 16α,17α-epoxypregnenolone.

3.3. Structure Determination of the Biotransformation Products. To characterize the structure of the product, the purified compounds were subjected to MS and NMR analyses. The ion peak $[M - H]^+$ at *m/z* 401 and $[M + Na]^+$ at *m/z* 425 in the ESI-MS spectra of compound **1** indicated a formula of $C_{27}H_{46}O_2$. The analysis of the ^{13}C spectral data showed 27 carbon signals (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5727631). A signal of δ143.5 and δ125.5 with sp^2 character suggested a double-doublet at C-5 and C-6 in DEPT data (Figure S2). This was affirmed by hydrogen shifts at δ5.28 (1H, t, $J = 2.4$ Hz) in the ^1H spectrum data (Figure S3). The carbon signal of δ71.4 (C-3) and 73.4 (C-7) showed that a hydroxyl group linked the A ring at C-3 and another hydroxyl group linked the B ring at C-7. We compared the shift value at δ125.5 (C-6) and 40.9 (C-8) of the product with δ121.6 (C-6) and 31.9 (C-8); β-OH can cause the chemical shift to increase greater than 4.8–7.4 downfield than α-OH. Based on this information from MS, ^1H NMR, and ^{13}C NMR, product **1** was characterized as 7β-hydroxycholesterol. This also agreed with the data in the literature [18]. The chemical structures of compound **1** were seen in Figure 4. The ^1H and ^{13}C NMR spectral data for the product was in Figures S14 and S15.

The analysis of the ESI-MS spectrum data of product **2** showed the ion peak $[M + H]^+$ at *m/z* 401.6. The analysis of the ^{13}C spectrum data of it showed 27 carbon signals. The data indicated a formula of $C_{27}H_{44}O_2$ (Figure S4). The signal of δ165.0 and δ126.2 with sp^2 character suggested a double-doublet at C-5 and C-6 in DEPT data (Figures S5 and S6). This was affirmed by hydrogen shifts at δ5.69 (^1H, s, H-6) of the ^1H spectrum data (Figure S7). The carbon signal of δ70.6 (C-3) showed that a hydroxyl group linked the A ring at C-3. Based on the information from MS, ^1H NMR, and ^{13}C NMR, compound **2** was characterized as 7-oxocholesterol. This was in accord with the data in literature [19]. The chemical structures of compound **2** were seen in Figure 4. The ^1H and ^{13}C NMR spectrum data of compound **2** was in Figures S14 and S15.

The analysis of the ESI-MS spectrum data of product **3** showed the ion peak $[M + H]^+$ at *m/z* 329.2 and $[M + Na]^+$ at *m/z* 351.2 and $[M + K]^+$ at *m/z* 367.1 in the ESI-MS spectra agreed with the formula $C_{21}H_{28}O_3$. The ^{13}C spectrum revealed 21 carbon signals (Figure S8): these were sorted by



(a) (b) (c)

Figure 3: TLC of the reaction solution of the steroids compounds. (a) TLC of the reaction solution of enzyme from strain SE-1 adding cholesterol. (b) TLC of the reaction solution of 16α,17α-epoxypregnenolone from strain SE-1. (c) 20-Droxyl-16α,17α-epoxypregn-1,4-dien-3-one being purified.

Table 1: Purification of the cholesterol 7β-hydroxylase from *Burkholderia cepacia* SE-1.

Step	Volume (mL)	Total protein[a] (mg)	Total activity[b] (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Ammonium sulfate	10	35.77	456.50	12.76	1.0	100.00
DEAE-cellulose	20	15.86	218.21	13.76	1.1	47.80
Sephadex G-200	10	5.76	107.69	18.70	1.5	23.59
PAGE[c]	0.5	0.58	21.59	37.22	2.9	4.73

[a]Protein concentration was determined by the method of Bradford [16] with bovine serum albumin as the standard.
[b]Cholesterol 7β-hydroxylase activity was assayed by measuring the quantity of 7β-cholesterol.
[c]PAGE: polyacrylamide gel electrophoresis.

DEPT experiments into $CH_3 \times 3$, $CH_2 \times 5$, CH × 8, and C × 5. The signal of δ155.1 (d, C-1), 127.7 (d, C-2), 124.1 (d, C-4), and δ168.3 with sp^2 character suggested a double-doublet at C-1 and C-2 and another double-doublet at C-4 and C-5 in DEPT data (Figures S9 and S10). The signal at δ186 showed a carbonyl group. These formed a conjugate system. This was affirmed by HSQC and HMBC experiments. The signal at δ7.01 (1H, d, J = 10.4 Hz), 6.22 (1H, dd, J = 10.4, 1.6 Hz), and 6.06 (1H, s) correlated with the δ155.1 (d, C-1), 127.7 (d, C-2), and 124.1 (d, C-4), respectively (Figure S11). The signal at δ7.01 (1H, d, J = 10.4 Hz) correlated with the δ168.3 (s, C-5) and 21.1 (q, C-19) and δ6.06 (1H, s) correlated with the δ127.4 (s, C-2), 33.1 (t, C-6), and 45.2 (s, C-10) in HMBC spectrum (Figure S12). The carbon signal of δ69.2 (d, C-20) showed a hydroxyl group at C-20. Based on the information from MS, ^{1}H NMR, and ^{13}C NMR, compound **3** was characterized as 20-droxyl-16α,17α-epoxypregn-1,4-dien-3-one. The TLC plate showed a red spot that indicated that 16α,17α-epoxypregn-1,4-dien-3-one has been transformed into 20-droxyl-16α,17α-epoxypregn-1,4-dien-3-one (Figure 3). The chemical structures of compound **3** were seen in Figure 4. The ^{1}H spectrum data of 3 was in Figures S13 and S14. The ^{13}C NMR spectrum data of 3 was in Figure S15.

3.4. Enzyme Purification. Table 1 summarizes the purification steps employed to purify the cholesterol hydroxylase. The TLC plate showed a deep blue spot that indicated that the enzyme had transformed cholesterol into 7β-hydroxyl cholesterol (Figure 3). The enzyme had a specific activity of 37.2 U/mg of protein at 30°C and pH 7.0. The retention time of 7β-hydroxycholesterol was 7.33 min. The standard curve is in Figure S16. The purified enzyme preparation gave a single band upon analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 5). Its molecular mass was estimated to be 80 kDa.

3.5. Amino Acid Sequence Analysis. The amino acid sequence of the purified enzyme was analyzed by MALDI-TOF/TOF. The MS data of pure enzyme is seen in Figure S17 and the MS/MS data of oligopeptides (Fr1–Fr3) in Figure S18 and those of oligopeptides (Fr4–Fr7) are seen in Figure S19. A trypsin digest of hydroxylase generated oligopeptides and the amino acid sequences of seven peptides (Fr1–Fr7) of the enzyme were determined. The sequences of Fr1–Fr7 were DWWPNQLNLNILHR, RFYENPAEFADAFAR, FYENPAEFADAFAR, HLFSYEW ELTK, IWLELSGGPNSR, VMNLDRFDLA, and SPAGAHQWVAK, respectively. These

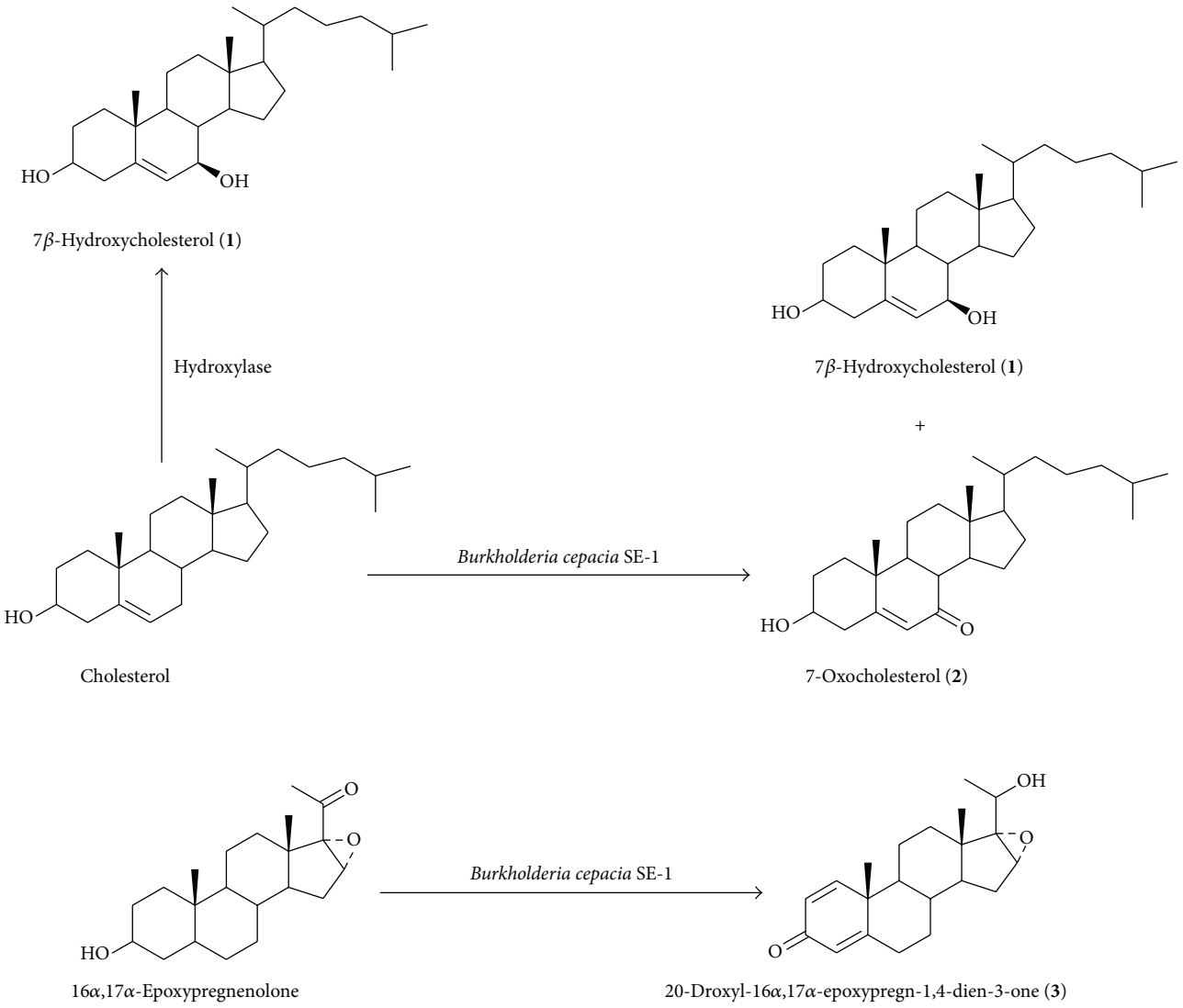


Figure 4: The chemical structures of cholesterol and 16α,17α-epoxypregnenolone and their hydroxylated substrates.

sequences are similar to those of catalases/peroxidases, for example, the catalase/peroxidase (*Burkholderia vietnamiensis* AU4i; the MS/MS data with Mascot accession number gi|543285001, protein sequence coverage: 24%), the heme catalase/peroxidase (*Burkholderia lata*, MS/MS data with Mascot accession number gi|78065290, 18%), catalase/hydroperoxidase HPI(I) (*Burkholderia cenocepacia*, MS/MS data with Mascot accession number gi|69880176, 12%), and catalase/hydroperoxidase HPI(I) (*Burkholderia ambifaria*, MS/MS data with Mascot accession number gi|493812374, 11%) (http://www.matrixscience.com/cgi/master_results.pl?file=../data/20130930/FTntOicSm.dat#Hit1).

4. Discussion

In the present study, the morphology of strain SE-1 showed different colony characteristics when grown on different media. It was pale yellow on the Beef Extract Peptone medium but was white and smaller in size on the screening medium. Doukyu reported that *Burkholderia cepacia* strain ST-200 produces an extracellular cholesterol oxidase which is stable and highly active in the presence of organic solvents. This cholesterol oxidase produces 6β-hydroperoxycholest-4-en-3-one from cholesterol. This oxidase contained bound flavin and was categorized as a 3β-hydroxysteroid oxidase, converting 3β-hydroxyl groups to keto groups. The molecular mass was 60 kDa. The enzyme is not inducible by cholesterol [20]. Under different nutritional conditions, *Burkholderia cepacia* strains can metabolize cholesterol by different pathways. Bacteria and fungi, especially of the genus of *Fusarium* and *Mucor*, that hydroxylate steroids at the C-7 position have been found. Kolek reported that dehydroepiandrosterone (DHEA), 5-androsten-3β,17β-diol, and 17α-methyl-5-androsten-3β,17β-diol were hydroxylated entirely at the 7α-axial, allylic position by *Fusarium culmorum*, which showed 7α-hydroxylase activity [21]. *Botryodiplodia malorum* showed the 7β-hydroxylation of DHEA and 15β,16β-methylene-dehydroepiandrosterone [22]. Morfin demonstrated that

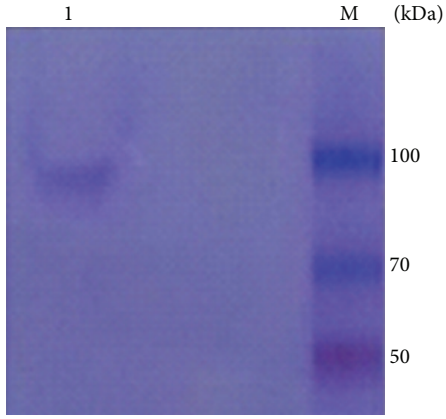


Figure 5: The SDS-PAGE. Line 1: hydroxylase; M: low molecular weight marker.

Fusarium moniliforme could almost entirely hydroxylate DHEA at the 7α-axial, allylic position but could not hydroxylate cholesterol [23]. It was confirmed that the 7α-hydroxylase was a cytochrome p450 enzyme (Cyp7b), which occurred in humans and animals [24]. This enzyme required $NADP^+$ as a coenzyme. Our experiments showed a requirement for NAD^+ in the generation of 7β-hydroxycholesterol. Appleby reported that they found cytochrome P450 in bacteria in 1967 [25]. Such proteins have oxidase activity, could catalyze some various exogenous substances, and converted into useful products to the microorganisms themselves. Cytochrome P450, as the terminal oxidase, mainly uses molecular oxygen, after one atom of oxygen combined with substrate, the other atom of oxygen, and hydrogen atoms to generate water provided by the NAD(P)H. This implied that our isolate, *Burkholderia cepacia* SE-1, contained hydroxylase.

5. Conclusions

Hydroxylation at position 7 of steroids with controlled stereoselectivity allows for the production of important pharmaceutical intermediates. Therefore, it was necessary that the microbial strains were screened which can transform steroids effectively. In our study, cholesterol and 16α,17α-epoxypregnenolone were transformed into useful products and hydroxylase was isolated from *Burkholderia cepacia* SE-1 successfully. These studies suggest that the steroid hydroxylase possesses 7β-site-selectivity and has latency value in production of the steroid medicine.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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