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Enzymatic-Based Hydrolysis of Digested Potato Peel Wastes by Amylase Producing Fungi to Improve Biogas Generation

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Abstract: Potato peel wastes are generated in high quantities from potato processing industries. They are pollutants to the environment, and they release greenhouse gases into the atmosphere. The present study assessed the potentiality of hydrolyzing potato wastes by amylase-producing fungi to improve biogas generation from potato peels through the anaerobic digestion process. Different fungal isolates were screened for amylase production on potato wastes, and the highest amylase producer was selected for optimizing the efficacy of producing amylases in high quantities to efficiently allow the conversion of potato organic matter into fermentable sugars that are utilized for the anaerobic digestion process. The best amylase producers were those derived from *Rhizopus stolonifer* (32.61 ± 0.89 U/mL). The highest cumulative methane yield from hydrolyzed potato peel was 65.23 ± 3.9 mL CH_4/g and the methane production rate was 0.39 mL CH_4/h, whereas the highest biogas yield from unhydrolyzed potato wastes was 41.32 ± 2.15 mL CH_4/g and the biogas production rate was 0.25 mL CH_4/h. Furthermore, it was found that the two combined sequential stages of anaerobic digestion (biogas production) followed by biodiesel production (enzymatic esterification) were the most effective, recording 72.36 ± 1.85 mL CH_4/g and 64.82% biodiesel of the total analytes. However, one-pot fermentation revealed that biogas yield was 22.83 ± 2.8 mL CH_4/g and the biodiesel extracted was 23.67% of the total analytes. The insights of the current paper may increase the feasibility of potato peel-based biorefinery through the biological hydrolysis strategy of potato wastes using eco-friendly enzymes.

Keywords: amylase; anaerobic digestion; biodiesel; biogas; potato peel wastes; *Rhizopus stolonifer*

1. Introduction

Potato crop is the third largest global crop [1] and is considered a vital nutritious food source in developing countries [1]. Recently, the growing development of the potato industry is attributed to the global increase in potato production and consequently warrants worldwide food security [2]. So, potato peel wastes are produced from potato processing in large quantities, whereas about 8% of potato weight is produced as waste material. It was reported that about 370 million tons of potato peel wastes are generated worldwide

annually [3] and the growing increase in these wastes from potato industries poses serious environmental and disposal problems. So, the abatement of potato wastes and converting them into green sustainable product including bio-oils and methane (CH_4) is becoming a major issue, whereas the major constituents of potato peels are starch, non-starch polysaccharide, protein, ash, and lipids [4]. Most of these solid organic wastes of urban areas are disposed of in landfills, which results in the loss of these areas and the release into the atmosphere of organic chemicals such as methane (CH_4) during anaerobic fermentation processes without them being properly utilized. Methane is a greenhouse gas with energetic value and has a global warming potential over 300 times more than CO_2 [5].

Nowadays, climatic changes, insufficiencies in energy storage and supply, oil price fluctuations, dependence on hydrocarbons for energy, and their critically dwindling availability have led to alternative ways to reduce energy requirements worldwide [6]. So, focusing on the sustainable economic use of this landfill organic waste is crucial right now for the production of future energy [6]. Therefore, anaerobic digestion (AD) can be adapted for the bioconversion of these organic wastes as raw material for biogas generation as a possible renewable and sustainable energy source [7]. Such a conversion is carried out using biochemical processes, such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis [4]. The anaerobic digestion process decreases air pollution because it is performed in a controlled closed system, which reduces the release of CH_4 into the atmosphere. However, the burning of the produced bio-CH_4 releases a green end-product (carbon-neutral carbon dioxide) which does not negatively affect the environment and does not cause global warming issues as it is the same as the CO_2 absorbed by plants for making their own food [8]. So, the organic waste treatment process for biogas production is advantageous from an energetic and environmental standpoint when compared to these organic materials being disposed of in landfills. Thus, anaerobic digestion techniques recycle organic wastes, producing clean, renewable energy sources (biogas) for heating and electricity generation or as a vehicle fuel [8,9]. For the enhancement of biogas production, the organic wastes must undergo one or more pre-treatments, such as thermal, mechanical, chemical, or biological treatment, before digestion to boost the substrate's biodegradability with minimum energy consumption [10]. The use of organic waste-degrading microbes or their enzymes might offer a cost-effective and ecologically sustainable method for producing simple and oligosaccharides, fatty acids, and amino acids that are involved in metabolic pathways for biogas production. Amylases are metalloenzymes that hydrolyze starchy substances into glucose, maltose, and maltodextrins and have proven to be crucial in many industrial processes including the food, pharmaceutical, fermentation, paper, textile, and detergent industries due to their prospective thermal and pH stability [11]. Different types of living organisms, such as bacteria, fungi, plants, and human, produce amylases for the breakdown of insoluble starch into soluble substances [11]. Fungi have greater potential for amylase production than bacteria from organic wastes. However, the advantages of growth-utilizing inexpensive renewable substrates, ease of enzyme extraction, greater stability of pH and temperature, and a reduced need for a cofactor make it a potential option for the commercial production of amylases [12]. So, the exploration of the treatment of potato peel wastes by amylase-producing fungi and the subsequent utilization of hydrolyzed potato peel for anaerobic digestion is crucial for the efficient operation and stability of the production systems [13].

The hydrolysis of potato peel wastes is an essential step that affects the fermentation and downstream operations during anaerobic digestion for biofuel generation. So, the main ideas of the current paper were to hydrolyze polymeric compounds of potato peel wastes and decrease the starchy material polymerization, which assists in the release of mono-sugars for the conversion of these waste materials into renewable green fuels [14]. For the enhancement and maximizing of the release of fermentable sugars, a zero-cost and eco-friendly tactic such as enzymatic hydrolysis is a top priority [15]. The zero-cost waste of potato peels contains a lot of starch and lignocellulose, which might be used to make bio-based products. The sustainable development of a circular green economy

would be supported by the valorization of potato peel wastes throughout a biorefinery concept, which would be advantageous for the production of several bioproducts and the reduction in the final waste residue. Anaerobic digestion or fermentation of potato peel wastes to generate biofuels is referred to as biological processing [5,16]. In addition to the biological processing of potato peel wastes, thermochemical processing using pyrolysis, hydrothermal liquefaction, and hydrothermal carbonization is carried out to decompose potato peel wastes into the desired bioproducts [17]. According to Karthikeyan et al. [18], thermal, physical, and chemical methods of treating food wastes for the anaerobic digestion process use more energy to mineralize and recover energy than biological methods, which are much more feasible, efficient, and economical.

As mentioned above, potato peel wastes contain some amounts of lipids, proteins, carbohydrates, and other polymeric substances. Compared to carbohydrates and proteins, lipids have the largest potential for the creation of biogas from food waste [19]. However, the presence of lipids in food wastes may lengthen the lag phase and prolong the anaerobic process's breakdown [20]. Moreover, the long-chain fatty acids generated by lipid hydrolysis may cause toxicity to fermentative bacteria, which in turn reduce the productivity of biogas production [20]. Hence, it is challenging to fully utilize anaerobic digestion to convert potato peel wastes to biogas. This seems to be one of the biggest challenges facing the fermentation of food waste to produce biogas. So, the technique described in the present work takes into account the remaining long-chain fatty acids and fatty acid esters contained in the discarded medium during anaerobic digestion. The yield of biofuel from potato peel wastes could be increased by integrating the production of biogas and biodiesel in a coupled system. Biodiesel can be synthesized chemically by using an acid/base catalyst or through an enzymatic transesterification/esterification process using a lipase enzyme. The transesterification/esterification process required the presence of an acyl acceptor in the form of short-chain alcohol [21].

The current study was carried out to (1) select a highly amylase-producing fungal isolate for hydrolyzing potato peel wastes; (2) improve the potato peel hydrolysis process as pre-treatment methods to maximize the efficiency of hydrolyzed potato peel wastes as feedstock for biogas production through an anaerobic digestion batch experiment; (3) assay the efficiency of hydrolyzed potato peel wastes for biogas production compared with unhydrolyzed wastes; (4) evaluate the potential production of biodiesel during the anaerobic digestion process from residual fatty acids and lipid contents of hydrolyzed potato peel wastes; and (5) calculate the energy content of biofuels produced from anaerobic digestion of potato peel wastes.

2. Results

2.1. Physico-Chemical Characteristics of Potato Peel Wastes

The obtained data revealed that the chemical analysis of potato peel wastes was as follows: 1 g of potato peel wastes contains 0.79 g water, and each 1 g dry matter contains 470 mg total sugars, 84.6 mg total protein, 54 mg total lipids, 268 mg reducing sugars and 36.18 mg free amino acids. These polymeric compounds could be used as effective raw materials for the production of biofuels.

2.2. Screening for Amylase Production by Fungi for Hydrolysis of Potato Peel Wastes

The data in Table 1 revealed that the tested twenty-six fungal isolates had different degrees of amylase production on potato peel wastes. The highest amylase production was estimated by Rhizopus stolonifer, recording 32.61 $\pm$ 0.89 U/mL, and the amylase-specific activity was 60.40 $\pm$ 2.74 U/mg protein.

Table 1. Extracellular amylase production, extracellular total protein, and lipase-specific activity of tested fungal isolates.

Test / Fungal Isolate	Amylase Enzyme Activity U/mL	Total Protein mg/mL	Amylase-Specific Activity U/mg Protein
Alternaria alternata	22.05 ± 0.86	1.14 ± 0.003	19.34 ± 1.62
Aspergillus			
A. awamorii	18.4 ± 1.64	0.96 ± 0.021	19.17 ± 0.94
A. candidus	13.85 ± 2.41	1.03 ± 0.04	13.45 ± 1.02
A. flavus	27.49 ± 0.54	0.91 ± 0.008	30.21 ± 2.42
A. flavus var *columnaris*	10.09 ± 0.73	1.07 ± 0.012	9.43 ± 1.07
A. ochraceus	9.51 ± 1.84	0.85 ± 0.007	11.19 ± 0.74
A. niger	30.65 ± 2.4	0.85 ± 0.031	36.06 ± 1.73
A. oryzae	28.09 ± 1.8	0.87 ± 0.027	32.29 ± 1.41
A. parasiticus	16.05 ± 2.12	0.9 ± 0.065	17.83 ± 0.83
A. tamari	9.16 ± 1.22	1.01 ± 0.008	9.07 ± 1.62
A. terreus	11.42 ± 1.06	0.87 ± 0.026	13.13 ± 2.01
Chaetomium globosum	17.27 ± 0.92	0.75 ± 0.032	23.03 ± 0.90
Cladosporium cladosporioides	10.05 ± 1.15	1.2 ± 0.041	8.38 ± 1.11
Emericella nidulans	14.16 ± 0.38	0.64 ± 0.007	22.13 ± 0.89
Eurotium amestelodami	7.15 ± 1.31	0.53 ± 0.042	13.49 ± 1.54
Fusarium			
F. solani	21.42 ± 2.16	0.96 ± 0.086	22.31 ± 0.95
F. verticillioides	26.59 ± 1.52	0.59 ± 0.022	45.07 ± 2.18
Penicillium			
P. chrysogenum	13.75 ± 2.51	0.81 ± 0.041	16.98 ± 0.66
P. citrinum	19.53 ± 0.87	0.73 ± 0.086	26.75 ± 1.07
P. digitatum	11.53 ± 2.3	0.92 ± 0.031	12.53 ± 1.09
P. funiculosum	21.43 ± 1.73	0.75 ± 0.051	28.57 ± 2.21
P. oxalicum	9.8 ± 1.1	0.83 ± 0.004	11.81 ± 1.67
P. purpurogenum	16.62 ± 1.58	1.1 ± 0.018	15.11 ± 2.14
Rhizopus stolonifer	32.61 ± 0.89	0.54 ± 0.013	60.40 ± 2.74
Scopulariopsis brumptii	7.57 ± 2.03	1.04 ± 0.098	7.28 ± 1.52
Trichoderma viride	17.92 ± 1.46	0.34 ± 0.021	52.71 ± 3.06

2.3. Enhancement of Amylase Enzyme Production on Potato Peel Wastes

The highest amylase-producing isolate was selected for the optimization of varying environmental and culture parameters for the enhancement of the amylase production on potato peel wastes. *Rhizopus stolonifer*, the highest amylase-producing isolate, is characterized by mycelia of aerial unbranched sporangiophores, stolons, and rhizoids. The tip of the sporangiophores is characterized by black sporangia that are rounded and filled with numerous nonmotile multinucleate sporangiospores (asexual reproduction units) (Figure 1).

a. Effect of incubation temperature

The impact of the incubation temperature of the culture on amylase production by *Rhizopus stolonifer* was studied at different incubation temperatures (15–45 °C). The obtained results in Figure 2 demonstrate that amylase productivity by *Rhizopus stolonifer* increased dramatically with an increase in the incubation temperature within the range of 15–30 °C. However, there was a drop in amylase production at temperatures above 30 °C. Therefore, the maximum enzyme production was estimated at 30 °C, recording 62.65 ± 1.16 U/mg protein.

b. Effect of pH on amylase production

Rhizopus stolonifer was incubated at variable pH values ranging from 4 to 11. The results plotted in Figure 3 indicate that the amylase yield increased with increasing the pH value of the culture medium between 4 and 6, and then the amylase production was decreased by increasing the pH value.

c. Effect of the incubation period

Rhizopus stolonifer was incubated for different incubation periods (2, 4, 6, 8, 10, 12, and 14 days) to investigate the effect of incubation time on the production of amylase. The data in Figure 4 show an association between amylase production by *Rhizopus stolonifer* with prolonging the incubation time, and the optimum period for the highest amylase production of 64.50 ± 0.96 U/mg protein was reached at 8 days of incubation and the amylase production started to decrease gradually (Figure 4).

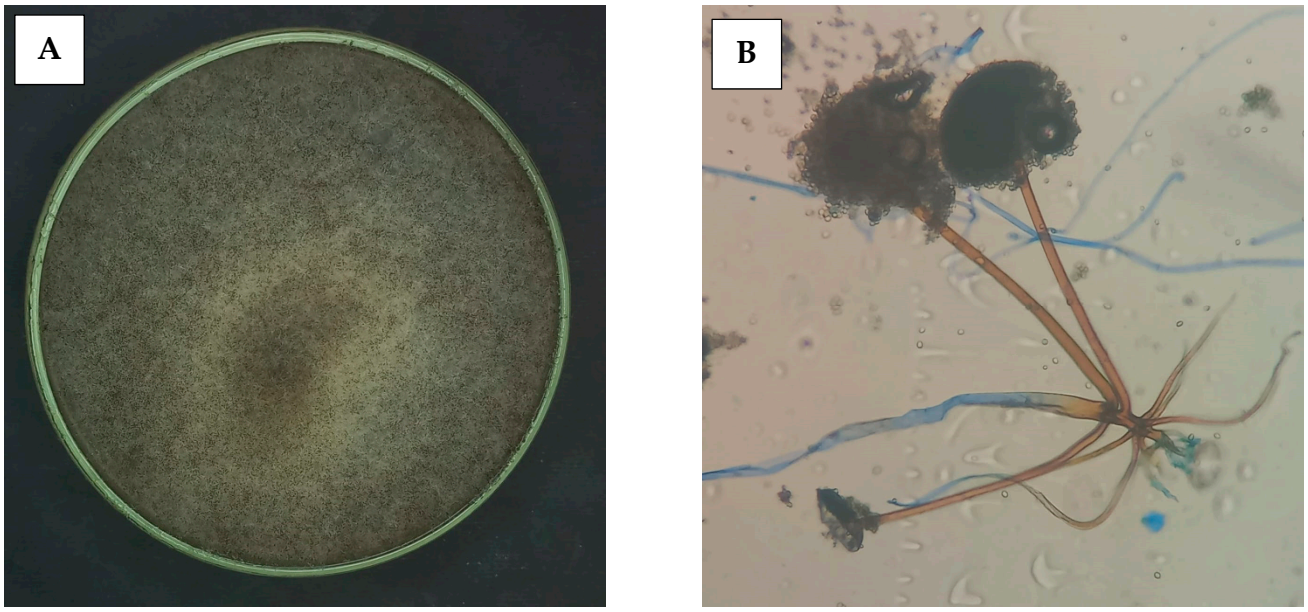

Figure 1. *Rhizopus stolonifer* grown on potato dextrose agar medium (**A**) and light microscope graph showing fungal sporangiophores, stolon, rhizoids, and sporangia filled with immotile sporangiospores (**B**).

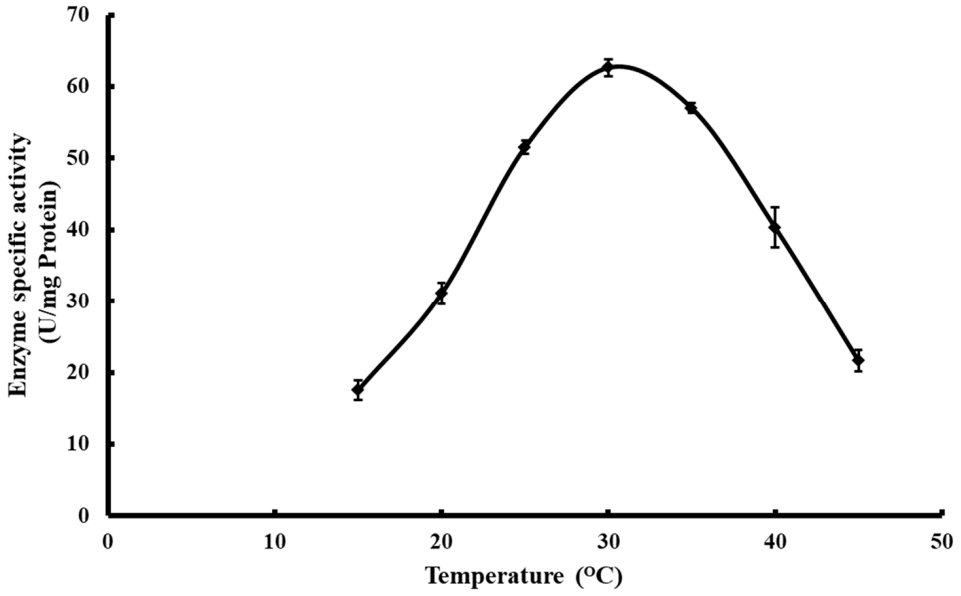

Figure 2. Effect of incubation temperature on amylase production by *Rhizopus stolonifer*. The triplicate means ± SD (vertical bars) are presented.

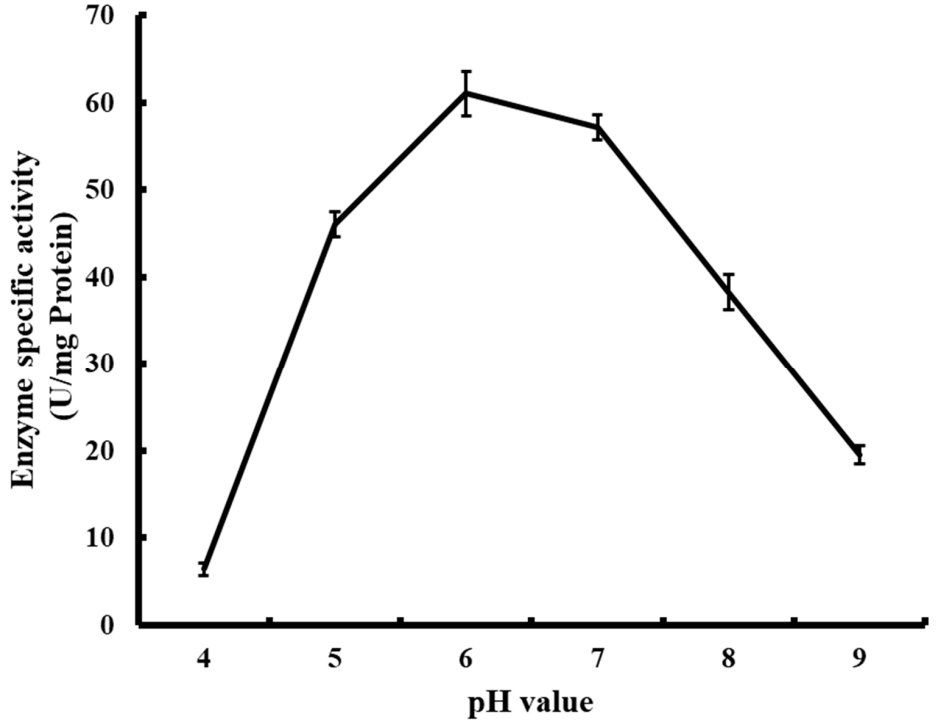

Figure 3. Effect of pH on amylase production by *Rhizopus stolonifer*. The mean of triplicate experiments is presented (mean ± SD (vertical bars)).

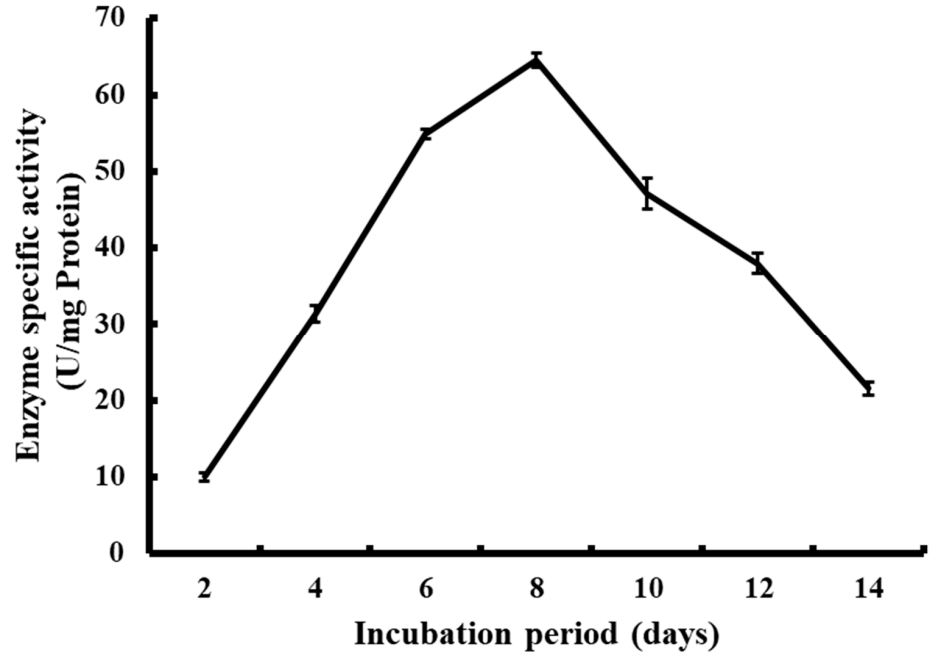

Figure 4. Effect of incubation period on amylase production by *Rhizopus stolonifer*. The mean of triplicate experiments is presented (mean ± SD (vertical bars)).

d. Effect of nitrogen source

The effect of different nitrogen sources on amylase production was investigated and the obtained results indicated that the tested fungal isolate *Rhizopus stolonifer* can make use of all the nitrogen sources. Among the tested nitrogen sources, yeast extract was the best substrate with the highest amylase production (66.70 ± 2.04 U/mg protein), followed by peptone, ammonium sulfate, ammonium nitrate, and sodium (Figure 5). So, yeast extract is

considered the best nitrogen source for the enhancement of amylase production on potato peel waste-containing medium.

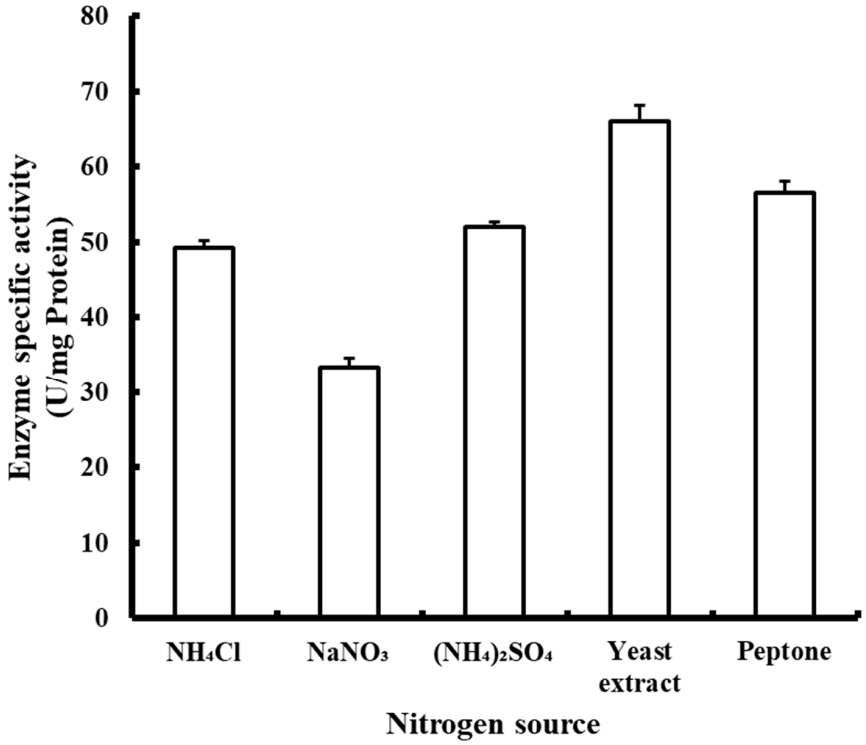

Figure 5. Effect of different nitrogen sources on amylase production by *Rhizopus stolonifer*. The mean of triplicate experiments is presented (mean ± SD (vertical bars)).

The impact of yeast extract on amylase production by *Rhizopus stolonifer* was investigated and the obtained data in Figure 6 show that the optimum concentration of the desired nitrogen source (yeast extract) was 3 g/L recording 79.15 ± 0.96, and by increasing the yeast extract concentration, amylase production decreased.

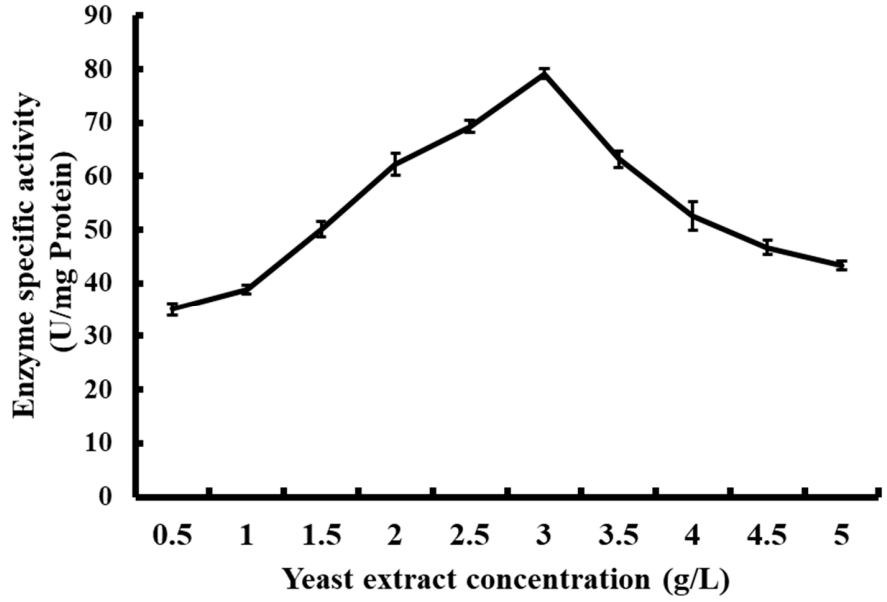

Figure 6. Effect of yeast extracts concentration on amylase production by *Rhizopus stolonifer*. The triplicate mean ± SD (vertical bars) is presented.

e. Effect of inoculum size

The data in Figure 7 reveal the impact of different fungal inoculum sizes (1–5 mL) on amylase enzyme production by *Rhizopus stolonifer* on potato peel wastes. The highest amylase production (91.17 ± 1.61) was achieved when an inoculum of 2 mL was used and then enzyme production was reduced with a higher inoculum dose. The lower inoculum dose may not be sufficient for starting fungal growth and enzyme production. However, the increase in inoculum size confirms fast fungal propagation and enzyme productivity.

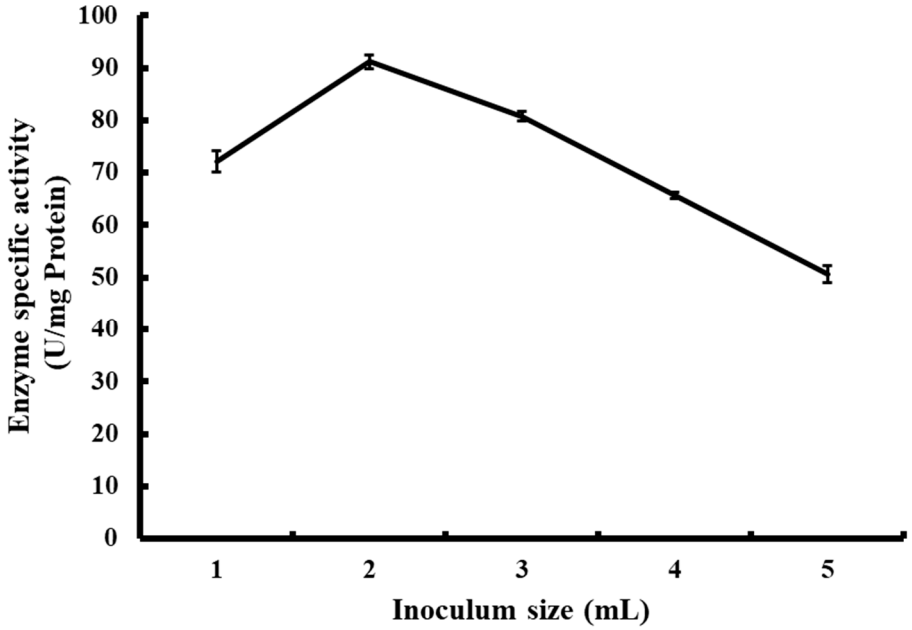

Figure 7. Effect of *Rhizopus stolonifer* inoculum size on amylase production. The triplicate mean ± SD (vertical bars) is presented.

2.4. Biogas Production from Potato Peel Wastes

In this study, the productivity of biogas production from hydrolyzed potato peel wastes by *Rhizopus stolonifer* and unhydrolyzed potato peel was assayed. After hydrolysis of potato peel wastes by *Rhizopus stolonifer*, the residual medium supernatant was utilized for biogas production from the fermentable sugars and long-chain fatty acids. The maximum cumulative methane yield was 65.23 ± 3.9 mL CH_4/g dry weight potato peel after 7 days of fermentation (Figure 8) and the methane production rate was 0.39 mL CH_4/h. In comparison, the highest methane production from unhydrolyzed potato wastes was 41.32 ± 2.15 mL CH_4/g and the methane production rate was 0.25 mL CH_4/h. Based on the data obtained, hydrolyzed potato peel wastes by *Rhizopus stolonifer* significantly enhance biogas production by 24.95% compared with biogas production from unhydrolyzed potato peel wastes through anaerobic digestion (Figure 8).

The GC/Ms analysis of hydrolyzed potato peel wastes after the hydrolysis process of potato peel wastes using *Rhizopus stolonifer* (the substrate for anaerobic digestion) and the residual medium after the fermentation process was performed. The obtained results revealed that the most common components recorded in the hydrolyzed potato peel supernatant were glyceryl tribehenate, ethyl iso-allocholate, 2,4-decadienal, and linoleic acid, recording 16.42, 10.06, 6.27, and 4.12% of the total analytes. The most prevalent components of the residual medium after the fermentation process were butanoic acid, pentanoic, ethyl iso-allocholate acid, and trilinolein, estimating 47.47, 8.57, 6.22, and 3.09% of the total analytes in addition to other free fatty acids (Table 2).

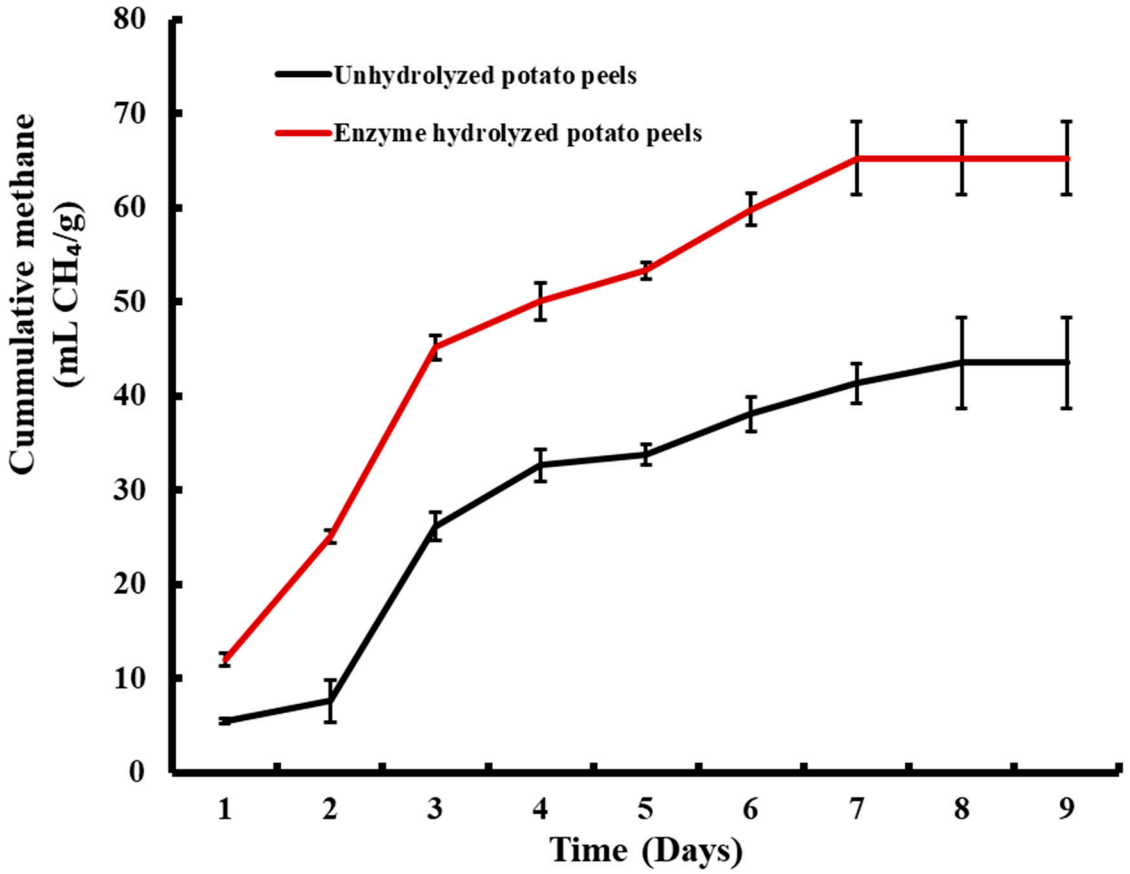

Figure 8. Cumulative biogas (methane) production from the hydrolyzed potato peel wastes and unhydrolyzed potato peels. The triplicate mean ± SD (vertical bars) is presented.

Table 2. Chemical characteristics of the culture medium before and after anaerobic digestion.

Analysis \ Medium	Spent Medium after Hydrolysis by *Rhizopus stolonifer*	Fermentation Medium after Anaerobic Digestion
Total carbohydrate (mg/L)	1347 ± 1030	5980 ± 340
Reducing sugars (mg/L)	4080 ± 90	6630 ± 670
Soluble amino acid (mg/L)	0.11 ± 0.0075	0.041 ± 0.0078
Total protein (mg/L)	48.09 ± 0.84	26.14 ± 2.31
Total lipids (mg/L)	794 ± 90	583 ± 10
Volatile organic acid (% of total)		
Linoleic acid	4.12	ND *
2,4-Decadienal	6.27	3.01
Oleic acid, 3-(octadecyloxy)	3.35	1.16
Ethyl iso-allocholate	10.06	6.22
Trilinolein	3.32	3.09
Glyceryl tribehenate	16.42	2.99
Butanoic acid	ND	47.47
Pentanoic acid	ND	8.57
Oleic acid	ND	1.39
Cyclopropanebutanoic acid	ND	2.10
Octadecanoic acid	ND	2.10
Hexadecanoic	ND	0.6
Cyclopropanedodecanoic acid	ND	0.62
Docosahexaenoic acid	ND	0.26
Docosahexaenoic acid	ND	0.44
Oleic acid, eicosyl	0.63	ND

* ND, not detected.

2.5. Coupled System for Biogas and Biodiesel Production during Anaerobic Digestion Process from Hydrolyzed Potato Wastes

Based on the analysis of the fermentation medium, different types of free fatty acids were detected that can be converted into biodiesel, so the current study detects the potentiality to perform a combined system for biogas production and biodiesel from the collected wastes of potato peel. A batch experiment of anaerobic digestion was performed for coupling biogas production from fermentable sugars of hydrolyzed potato wastes and biodiesel production from the detected long-chain fatty acids to maximize the utilization of the organic matter of potato peel wastes. So, the anaerobic digestion in the current study was performed in two sets: (a) the one-pot fermentation process through the addition of methanol during the anaerobic digestion process and (b) sequential two-stage acidogenesis and methanogenesis for biogas production followed by the lipase transesterification/esterification process for biodiesel production.

Interestingly, *Rhizopus stolonifer* exhibited a high potentiality for lipase production, recording 64.96 ± 2.89 U/mL, and lipase-specific activity, 141.22 ± 6.04 U/mg protein. The produced lipase converts total lipids into free fatty acids that can be esterified into fatty acid methyl esters (biodiesel). In addition, the obtained results of the analysis of hydrolyzed potato peel revealed the presence of a significant amount of the long-chain organic acids (Table 2) which were metabolized by acidogenic and methanogenic bacteria to produce biogas, as well as the data obtained from the analysis of the supernatant after anaerobic digestion revealed the release of free fatty acids in the residual medium which is considered a potent feedstock for biodiesel production through the esterification/transesterification process.

The obtained results showed that in the two sequential stages of the anaerobic digestion stage for the biogas and esterification process, an increase in biogas production was revealed compared to the one-pot fermentation process (Figure 9). However, the highest biogas production from the two stages (without the addition of methanol during anaerobic fermentation) was 72.36 ± 1.85 mL CH_4/g, recorded at 8 days, and the production of biogas increased with fermentation time until the 8th day and then biogas production stopped. On the other hand, the highest biogas production from the one-pot fermentation method (with the addition of methanol during anaerobic digestion) recorded at 6 days from the start of the experiment estimated a biogas yield of 22.83 ± 2.8 mL CH_4/g.

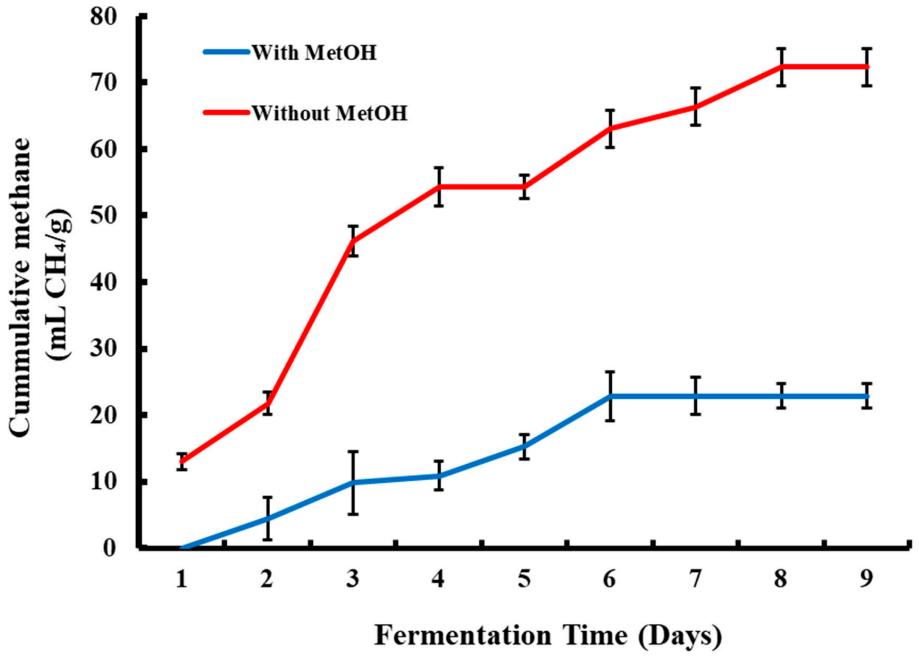

Figure 9. Cumulative biogas (methane) is produced from one-pot fermentation (with the addition of methanol) and sequential two-stage fermentation. The triplicate mean ± SD (vertical bars) is presented.

2.6. Energy Content

A. input energy

Total input energy = total energy consumed for sterilization by autoclaving + incubation in the shaker incubator + stirring of bottles for anaerobic digestion + esterification process.

So, total input energy = 2250 + 3960 + 166.32 + 95.04 = 6471.36 KJ

B. output energy

Energy content from biogas and biodiesel was calculated from combustion energetics that were estimated from the bond energies of reactants and products as in the following:

$$\Delta H = \Sigma BE \text{ (bonds broken)} - \Sigma BE \text{ (bonds formed)}$$

where BE is the bond energies.

Energy content from biogas:

$$CH_4 + 2O_2 \rightarrow 2H_2O + CO_2$$

$$\Delta H = (1640 + 988) - (1598 + 1840)$$

$$\Delta H = -810 \text{ KJ}$$

Energy content from biodiesel:

$$C_{19}H_{36}O_2 + 54\ 1/2O_2 \rightarrow 18\ H_2O + 19\ CO_2$$

$$\Delta H = (21{,}611 + 13{,}117.5) - (27.164.5 + 17{,}480)$$

$$\Delta H = -9916 \text{ KJ/mole}$$

So, the gross energy from the sequential stage of biogas and biodiesel (output energy) was 10,726 KJ/mole:

$$\text{Net energy} = \Sigma_{\text{output energy}} - \Sigma_{\text{input energy}}$$

$$\text{Net energy} = 10{,}726 - 6471.36 = 4254.64 \text{ KJ}$$

3. Discussion

3.1. Physico-Chemical Characteristics of Potato Peel Wastes

Potato is considered one of the most abundant crops worldwide, and consequently, large quantities of potato peel wastes are generated from potato processing industries, which present a global hazard to the environment. Nevertheless, potato peel wastes are a potential renewable feedstock for the production of valuable, sustainable green products due to their availability, superb nutritional qualities, and low cost. So, the current study presents state-of-the-art technology for the improvements in the biological treatment of potato peel wastes to maximize the generation of biofuel output. Additionally, the strategies of pre-treatments and hydrolysis, followed by anaerobic digestion showed considerable potential for the manufacturing of biofuels that provide sustainable alternatives to petroleum fuels and mitigate environmental issues.

Understanding the chemical components of potato peel wastes is crucial to ensure the most beneficial usage of these wastes. The chemical analysis of potato peel wastes stated the presence of different biomolecules, including total sugars, total protein, total lipids and reducing sugars, and free amino acids. Interestingly, recent studies revealed that potato peel wastes are composed mainly of polymers of lignocellulosic materials, starch, protein, and lipids as well as amounts of glycoalkaloids and phenols [4]. As well, the starchy material, consisting of about 35% of the potato peel wastes, has a significant potential for being converted into added-value green products by microorganisms. However, a wide range of microbes cannot directly ferment starch during the fermentation process; so, starch

hydrolysis by microbial enzymes is necessary to produce fermentable sugars [4] that can be utilized through anaerobic digestion to produce biofuels. Furthermore, proteins and lipids in potato peel wastes can be exploited for anaerobic digestion, although proteins and lipids do not have the same biological conversion potential as sugars [22]. To remove these structural barriers for anaerobic digestion, a pre-treatment process is essential for maximizing biogas production. So, the polymeric compounds of potato peel wastes must undergo a subsequent hydrolysis process to be transformed into monomeric sugars that can then be used to create bioproducts [23].

3.2. Screening for Amylase Production by Fungi for Hydrolysis of Potato Peel Wastes

The data in Table 1 revealed that all the tested fungal isolates revealed various capabilities for amylase production on potato peel wastes and the highest amylase producer fungal isolate was *Rhizopus stolonifer*, recording amylase productivity of 32.61 ± 0.89 U/mL and amylase-specific activity of 60.40 U/mg protein. There were several studies conducted to utilize agricultural organic wastes for amylase enzyme production by fungi, whereas Ahmed et al. [24] investigated amylase enzyme production from different agro-wastes and the maximal activity was reported by pomegranate peel. Different fungal isolates showed high efficacy to produce an amylase enzyme, such as *Aspergillus*, *Penicillum*, and *Rhizopus* [25]. As it was reported, fungal amylases showed a potential application and are attracting interest due to the mild operating parameters, decreased by-product production, lower refining, and recovery costs [11]. In addition, many investigations on fungal amylase production, particularly in developing nations, were performed mainly on *Aspergillus* spp. and *Rhizopus* spp. because of their abundance and simple nutritional needs. Interestingly, Benabda et al. [26] stated that *Rhizopus oryzae* grown on the solid-state fermentation of humidified bread waste produced amylase production with activity of 100 U/g.

3.3. Enhancement of Amylase Enzyme Production on Potato Peel Wastes

One of the most crucial methods for producing appropriate enzymes in large quantities to satisfy industrial demand is dependent on the optimization of the varying parameters, including the media [27]. For this reason, we have worked on the crucial parameters that are likely to influence optimization techniques for the highest amylase productivity.

The obtained data revealed that the optimum conditions for amylase production by *Rhizopus stolonifer* were 30 °C, a pH value of 6, and an incubation time of 8 days, using yeast extract (3 g/L) and an inoculum size of 2 mL/25 mL. Vidyalakshmi et al. [28] stated that incubation temperature is considered one of the significant parameters which considerably influences amylase production [29]. Interestingly, many studies on amylase production using different fungal isolates were performed with mesophiles that grow best at temperatures between 25 and 37 °C [30]. However, Sindhu et al. [31] stated that at incubation temperatures above 45 °C, there was a negative effect on the metabolic activities of the microorganism, and consequently reduced fungal growth and enzyme productivity. Furthermore, the hydrogen ion concentration of the culture medium has an important role in inciting morphological alternations and variations in fungal mycelia and consequently in the production of enzymes [32]. Rizk et al. [33] found that the activity of amylase enzyme was enhanced by increasing the pH value up to pH 6.5. Singh et al. [33] stated that the activity amylase enzyme produced by *Aspergillus fumigatus* NTCC1222 was enhanced with the increase in the incubation period, and the maximum activity was observed on the sixth day, and then the enzyme activity was decreased. Benabda et al. [34] reported that the highest amylase activity by *Rhizopus oryzae* growing on bread waste was achieved after 5 days of fermentation, recording 100 U/g, and then the enzyme activity dropped drastically. As well, Ahmed et al. [35] conducted a study for the optimization of alpha-amylase production by fungi using organic nitrogen source corn steep. Interestingly, it was found that the lower inoculum dose may not be sufficient for starting fungal growth and enzyme production. However, the increase in inoculum size confirms fast fungal propagation and enzyme

productivity. Up to a definite inoculum limit, enzyme productivity was lower because of the exhaustion of available nutrients, resulting in a decline in metabolic activity [36].

3.4. Biogas Production from Potato Peel Wastes

Interestingly, potato peel wastes have been intensively reported as a cost-effective feedstock to produce different green products, including biofuels, biochemical, enzymes, biopolymers, and organic acids. Awogbemi et al. [37] reported on the possibility of the conversion of potato wastes to produce biofuels. In the current study, biological conversion of potato wastes was performed by hydrolysis tactics to improve potato peel biodigestibility, hydrolysis, and consequently fermentative generation of different sustainable green bioenergy through an anaerobic digestion process. The obtained data revealed that the cumulative methane produced from hydrolyzed potato peel wastes by *Rhizopus stolonifer* and unhydrolyzed potato peel was 65.23 ± 3.9 mL CH_4/g and 41.32 ± 2.15 mL CH_4/g, respectively. However, the biogas production rate from enzymatically hydrolyzed potato peel wastes and unhydrolyzed potato peel was 0.39 mL CH_4/h and 0.25 mL CH_4/h, respectively. Consequently, the obtained data revealed that enzymatically hydrolyzed potato peel wastes significantly enhance biogas production through anaerobic digestion by 24.95% compared to unhydrolyzed potato peel wastes (Figure 7). Interestingly, the analysis of the residual medium of enzymatically hydrolyzed potato peel wastes (the substrate for anaerobic digestion) using a GC/Ms analysis of also the residual medium after the anaerobic digestion process revealed the presence of various free fatty acids and fatty acid esters. The most common compounds recorded in the enzymatically hydrolyzed potato peel supernatant by *Rhizopus stolonifer* were glyceryl tribehenate, ethyl iso-allocholate, 2,4-decadienal, and linoleic acid. However, the most common components of the residual medium after the fermentation process were butanoic acid, pentanoic acid, ethyl iso-allocholate, and trilinolein. The obtained results after anaerobic digestion did not reveal the presence of acetic or propionic acid and this may be due to the utilization of these short-chain acids for biogas production during acidogenesis and methanogenesis processes.

Biogas is primarily generated from the anaerobic digestion of organic potato wastes through a four-stage process. The first stage involves the hydrolysis process to hydrolyze complex materials into simple ones and then the acidogenesis process to convert simple compounds into long-chain acids. Then, the acetogenesis process converts long-chain acids into acetic acid and is followed by methanogenesis for the breakdown of acetic acid formed into methane or through the reduction in carbon dioxide [7]. Potato peel wastes contain a high number of biodegradable materials that are considered competent resources for biogas production [38]. Achinas et al. [39] stated that the maximum biogas production (485.4 mL/gVS) was obtained from acid pre-treatment of potato wastes/cow manure through anaerobic co-digestion. In addition, the methane output from combined potato wastes and pig manure (50:50 ratio) was improved by 25% compared to feedstocks that were mono-digested [16].

3.5. Coupled System for Biogas and Biodiesel Production during Anaerobic Digestion Process from Hydrolyzed Potato Wastes

The current study stated that *Rhizopus stolonifer* exhibited high lipase activity (64.96 ± 2.41 U/mL) and lipase-specific activity (141.22 ± 3.06 U/mg protein). So, *Rhizopus stolonifer* may provide high potential as a biocatalyst to convert free fatty acid and fatty acid esters in biodiesel. Interestingly, the obtained data from the GC/Ms analysis of hydrolyzed potato peel revealed the presence of a significant amount of free fatty acids in the residual medium after the fermentation process of anaerobic digestion (biogas production process) which is considered a potent feedstock for biodiesel production.

The obtained data revealed that the coupled two stages for the anaerobic digestion stage (biogas production) and esterification/transesterification process (biodiesel production) exhibited an increase in biogas production compared to the one-pot fermentation process, recording 72.36 ± 1.85 mL CH_4/g and 22.83 ± 2.8 mL CH_4/g, respectively. The

observed reduction in biogas production using one-pot fermentation may be due to the inhibitory effects of methanol [21]. However, the yields of biodiesel produced from the coupled two stages and one-pot fermentation were 64.82% and 23.67% of the total analytes. Interestingly, it was reported that the anaerobic digestion process also generated a variety of other biochemicals as fermentation metabolites. Among those, volatile fatty acids and organic acids are the most significant biochemicals obtained by potato wastes as feedstock [40].

The sustainability of biogas is supported by multiple economic analyses [41], not only in aspects of energy–environmental sustainability but also in regard to economic sustainability [42]. For instance, the production of biofuels from potato peel wastes for sale or the utilization of the produced effluent for agriculture as a biofertilizer (as a good and cheap organic fertilizer) [43]. It is worth noting that the indirect economic benefits from biogas generation can have higher economic benefits leading to more employment opportunities, especially in rural communities [44]. As stated by Bond and Templeton [12], the economic advantages of deploying biogas technology in an optimal cost-efficient anaerobic digestion system can positively impact the environment and society [45]. These impacts include enhanced sanitation, a reduction in the prevalence of infections and disease transmission, low-cost electricity for cooking and lighting, low-cost fertilizers, enhanced crop yields, improved living conditions for existing plants, improved air quality, and reduced greenhouse gas emissions [44,45].

4. Materials and Methods

4.1. Physico-Chemical Analysis of Potato Peel Wastes

Potato peel wastes were collected from local restaurants at Assiut governorate packed in sterilized polyethylene bags and transported to the laboratory and stored at a temperature of −20 °C until used for enzymatic assay. The physico-chemical characteristics of the collected potato peel wastes were analyzed. Protein content was assessed according to Lowry et al. [46]. Total lipids were assayed by phospho-vanillin reagent method [47]. Total sugars were assessed by the anthrone-sulfuric method [48]. Free amino acids were assayed according to Muting and Kaiser [49]. Reducing sugars were assessed by the dinitrosalicylic acid method [50].

4.2. Microorganisms and Preparation of Inoculum

A total of 28 fungal isolates isolated from potato-cultivated soils in Upper Egypt and preserved in potato dextrose agar (PDA) medium at 4 °C were employed in this investigation. The morphological characteristics of fungal isolates were employed for their identification using the following keys of Pitt [51], Raper and Fennell [52], and Domsch et al. [53]. For the preparation of fungal inoculum, PDA culture plates of fungi grown at 25 °C were used that were 7 days old. Scraped fungal hyphae from the surface of PDA plates were combined with sterile distilled water, and 1 mL of a homogeneous mixture of fungal spore suspension was utilized as fungal inoculum.

4.3. Screening for Amylase Production by Fungi on Potato Peel Wastes

A total of 28 fungal isolates were grown in 100 mL flasks containing 25 mL sterile basal salt medium containing (g/L) $NaNO_3$, 2; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005; pH 6.8. Each conical flask was supplemented with 0.5 g potato peel wastes as the carbon source for investigation of amylase activity. The culture media were autoclaved and then inoculated with 1 mL fungal spore suspension (1×10^6 CFU), and then incubated at 28 ± 2 °C for 7 days in shaking incubator (Environ 3597-1, LabLine Instruments, Melrose Park, IL, USA). After the incubation period, the fungal cultures were centrifuged, and the fungal supernatant was used to assay amylase activities.

4.4. Determination of Amylase Activity

Amylase activity was assayed by assessing the amount of reducing sugars produced from hydrolyzed potato peel wastes. The reaction mixture contained 900 µL of 1% soluble starch in 50 mM citrate buffer (pH 5) and 100 µL mL of fungal supernatant (crude enzyme) and then incubated at 50 °C for 10 min, then the reaction was stopped by adding 1 mL of dinitrosalicylic acid reagent, and incubated at 90 °C for 10 min to assay the produced reducing sugars. The resulting color was assessed using a UV–visible spectrophotometer at 540 nm. A unit of amylase activity is the quantity of crude enzyme that, under the assay conditions, releases 1 µmol of reducing sugars per min. Enzyme-specific activity was estimated as an enzyme unit per mg protein and the culture's protein content was measured according to Lowry et al. [46].

4.5. Enhancement of Amylase Enzyme Production on Potato Peel Wastes

The high amylase-producing fungal isolate *Rhizopus stolonifer* ASU 23 was selected for optimization of the cultural and environmental conditions for achieving higher potato peel waste hydrolysis into simple sugars by amylase enzyme.

a. Effect of incubation temperature

For maximizing amylase production, *Rhizopus stolonifer* ASU 23 was grown on the basal salt medium containing (g/L) $NaNO_3$, 2; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005; pH 6.8, and incubated at different incubation temperatures (15, 20, 25, 30, 35, 40, and 45 °C) for 7 days; at the end of the incubation period, the amylase activities were assayed as described previously (Section 4.4).

b. Effect of initial pH value

Rhizopus stolonifer ASU 23 was grown on the basal salt medium containing (g/L) $NaNO_3$, 2; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005, and incubated at the optimum temperature (30 °C) for 7 days. Different initial pH values (4, 5, 6, 7, 8, and 9) were investigated, and the initial pH of the culture medium was adjusted by using either 100 mM HCl or 100 mM NaOH. After 7 days, the amylase activities were assayed as described previously.

c. Effect of different incubation period

Different incubation periods were investigated for the highest amylase production through the growing of *Rhizopus stolonifer* ASU 23 on the basal salt medium containing (g/L) $NaNO_3$, 2; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005, at the optimum temperature (30 °C) and pH value (6). At the end of the tested incubation period, the amylase activities were assayed as described previously.

d. Effect of different nitrogen sources

Rhizopus stolonifer ASU 23 was cultivated on the basal salt medium containing (g/L) KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005, supplemented with different nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$, peptone, and yeast extract) equivalent to 1 g $NaNO_3$ were tested by cultivation at the optimum temperature (30 °C), pH value (6), and incubation period 8 days. After 8 days of incubation, the amylase activities were assayed as described previously.

e. Effect of nitrogen concentration

Different yeast extract concentrations of "the optimum nitrogen source" (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 g/L) were investigated for the highest amylase production by *Rhizopus stolonifer* grown on basal salt medium containing (g/L) KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005, at the optimum temperature (30 °C) and pH value (6) and incubation period 8 days. The amylase activities were assayed as described previously.

f. Effect of different inoculum size

Different inoculum sizes of *Rhizopus stolonifer* (1, 2, 3, 4, 5 mL/25 mL) were assayed for investigation of the highest amylase production in culture medium containing (g/L) yeast extract, 3; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.005, pH value (6), and incubated at the optimum temperature (30 °C). After incubation period 8 days, the fungal supernatant was collected by centrifugation and the yield of amylase produced was assessed as described previously.

4.6. Biogas Production from Potato Peel Wastes

a. Preparation of substrate

A batch experiment was employed to investigate and compare the productivity of biogas production from hydrolyzed and unhydrolyzed potato peel wastes. *Rhizopus stolonifer* was grown on the previously described medium containing potato peel wastes (2%) under the optimized enzymatic saccharification conditions. The potato peel wastes were hydrolyzed into fermentable sugars and then the fungal supernatants containing hydrolyzed sugars were collected and the pH was adjusted to 8 and used as a culture medium for biogas production via an anaerobic digestion process. Furthermore, unhydrolyzed potato peel wastes were assayed for their efficiency in biogas production.

b. Fermentation process for biogas production

Sewage sludge collected from a sewage plant in Assiut City, Egypt, was used as an inoculum for biogas production from potato peels via batch anaerobic digestion process. In order to perform the fermentation, in 500 mL glass bottles (with 50 mL head space), 440 mL of hydrolyzed or unhydrolyzed potato peels medium was mixed with 10 mL of sewage sludge (17.25 g organic matter per liter fermentation medium). The bottles were then tightly closed with rubber septa and anaerobically purged with nitrogen. Bottles were placed on a hot plate with a stirrer and stirred with a magnetic stirrer at 30 °C (room temperature) for 14 days at 120 rpm. The gas generated during anaerobic digestion was collected and measured in a cylinder immersed in water and connected to a 2 M NaOH solution to absorb carbon dioxide (water displacement method) and expressed as mL methane per gram dry weight of potato peel waste. The biogas generated was analyzed by a gas chromatography apparatus (Shimadzu GC-2014, Shimadzu Scientific Instruments Incorporated, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and Shin Carbon packed column (ST 80/100 2 m, 2 mm ID), and all gas volumes were reported.

4.7. Coupling Biogas and Biodiesel Production during the Anaerobic Digestion Process

Based on the analysis of potato peel wastes, lipid contents and free fatty acids were detected as biomolecules, so these molecules could be converted into biodiesel through esterification and transesterification process in the presence of a catalyst (biocatalyst lipase) and acyl acceptor (short chain alcohol, e.g., methanol). So, this experiment was designed to investigate the potentiality of *Rhizopus stolonifer* ASU 23 to produce lipase enzyme, as well as the lipase esterification and transesterification process being performed in one-pot fermentation process during biogas production and in two sequential stages (biogas production stage followed by methanolysis of free fatty acids).

a. Determination of lipase activity

Rhizopus stolonifer ASU 23 was grown in 100 mL Erlenmeyer flask containing 25 mL of liquid medium containing (g/L) peptone, 15; yeast extract, 5; NaCl, 2; $MgSO_4$, 0.4; K_2HPO_4, 0.3; KH_2PO_4, 0.3, and the culture medium was supplemented with tween 80, 10 mL for lipase induction [54]. Three replicates of conical flasks were incubated for 7 days at 37 °C under shaking at 120 rpm. The lipase activities were assayed in the culture supernatant with *P*-nitrophenylpalmitate according to Prazeres et al. [55]. A 1 unit lipase (U/mL) was expressed as the amount of enzyme to liberate 1 µmol nitrophenol/min.

b. Lipase esterification/transesterification process

i. One-pot fermentation process The batch anaerobic digestion process was performed using stopper glass bottles (500 mL) for dual production of biogas and biodiesel from potato peel wastes. Each glass bottle contained 430 mL hydrolyzed potato peel, 10 mL of sewage sludge (2.3% of the fermentation medium), and 10 mL methanol (acyl acceptor) and then incubated at 30 °C for 14 days. The produced biogas was collected and measured as described previously, whereas the produced biodiesel (fatty acid methyl esters) was collected in n-hexane layer and evaluated using GC/MS (Model: DPC-Direct Probe Controller (DPC-20451), Thermo Scientific, Waltham, Massachusetts, USA) at the Chemistry Department, Faculty of Science, Assiut University. GC/MS was equipped by a capillary column TG-5MS (30 m, 0.25 mm i.d., 1 μm film thicknesses). The oven temperature was 80 °C for 5 min, then increased at the rate of 10 °C/min to 150 °C and 200 °C for 10 min each, and finally the oven temperature was raised at an increasing rate of 5 °C/min to 250 °C for 13 min. The injector temperature was 250 °C and the detector temperature was 300 °C. The percentage of FAMEs yield was estimated by comparison with the peak area of internal standards at the specific retention time [27].

ii. Sequential coupled stages A sequential coupled-stage esterification/transesterification process comprising hydrolysis and methanolysis processes was conducted to alleviate methanol (acyl acceptor) inhibitory effect on anaerobic digestion and enzyme esterification reaction. Firstly, the anaerobic digestion process of potato peel wastes was performed without the addition of methanol. After the incubation period and collection of the produced biogas, the residual free fatty acids in the fermentative medium produced in the first stage were collected for the next stage. Secondly, the collected fermentative medium (10 mL) was taken through a methanolysis step in a screw-top bottle, and 2 mL of methanol was added to the reaction mixture to produce biodiesel through the esterification process. The bottles were incubated at 60 °C while stirring overnight. As previously mentioned, the generated biodiesel (fatty acid methyl esters) was analyzed using GC/MS.

4.8. Calculation of Total Energy Content (Input and Output Energy)

A. input energy

The gross energy utilized for completing the full process includes the energy used by autoclaving, incubation of flasks, and stirring of bottles for anaerobic digestion.

Total input energy = total energy consumed for sterilization by autoclaving + incubation in the shaker incubator + stirring of bottles for anaerobic digestion + esterification process.

$$\text{Total input energy} = 2250 + 35.28 + 11.88 + 20.16 = 2317.32$$

B output energy

The gross energy generated from biogas and biodiesel was calculated from combustion energetics according to the following equations:

$$CH_4 + 2O_2 \rightarrow 2H_2O + CO_2 \text{ (For biogas)}$$

$$C_{19}H_{36}O_2 + 53\ 1/2O_2 \rightarrow 18\ H_2O + 19\ CO_2 \text{ (For biodiesel)}$$

Energy content from biogas and biodiesel was calculated from combustion energetics that were estimated from the bond energies of reactants and products as follows:

$$\Delta H = \Sigma BE \text{ (bonds broken)} - \Sigma BE \text{ (bonds formed)}$$

where BE is the bond energies.

$$\text{Net energy} = \Sigma_{\text{output energy}} - \Sigma_{\text{input energy}}$$

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