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Valorization of Food Waste Slurry as Potential Candidate for Lipid Accumulation: A Concept of Oleaginous Bio-Refinery

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Abstract: In the current state of huge waste production and energy crisis, there is a need to find additional alternate energy resources and options for waste management. The present study was designed to measure the potential of different fruit wastes to serve as substrate for lipid accumulation in oleaginous bacteria. For this purpose, three novel bacterial strains (AF3, KM1 and KM10) isolated from the crude oil samples were systematically compared for their lipid accumulation potential using three types of waste including orange waste (OW), mango waste (MW) and apple waste (AW). Using waste as sole substrate, it was observed that maximum lipid accumulation by each strain was above 20%, which confirms that the bacteria belong to the oleaginous group. However, each bacterial isolate represented differential accumulative capacity with varying organic matter removal efficiency. Maximum lipid accumulation was achieved by KM10 (>25%) with AW as substrate, and KM1 (>24%) with MW as substrate; however, AF3 represented only 21% lipid accumulation using AW as substrate. Similarly, the maximum removal efficiency was recorded for KM10 in AW, followed by OW, where >60% and >50% of volatile solids (VS) removal, respectively, were achieved over the period of 7 days of incubation. This showed that the oleaginous strains also exhibit excellent waste treatment efficiency. The 16s RNA gene sequencing results showed that these KM1 and KM10 strains were *Serratia surfactantfaciens* and *Serratia liquefaciens*. In the end, a circular economy model was presented to highlight the significance of the mechanisms, which offers dual benefits over the linear economy model. Overall, the findings of the present study revealed that the novel oleaginous strains not only provide considerable lipid accumulation, but are simultaneously capable of low-cost waste treatment.

Keywords: oleaginous bacteria; biolipids; food procession waste; biorefinery; circular economy

Citation: Qadeer, S.; Khalid, A.; Mahmood, S.; Saleem, A.R.; Anjum, M. Valorization of Food Waste Slurry as Potential Candidate for Lipid Accumulation: A Concept of Oleaginous Bio-Refinery. *Fermentation* **2023**, *9*, 163. https://doi.org/10.3390/fermentation9020163

Academic Editors: Aijuan Zhou, Cristiano Varrone and Zhangwei He

Received: 4 January 2023
Revised: 5 February 2023
Accepted: 8 February 2023
Published: 10 February 2023

1. Introduction

Fossil fuels and their subsequent challenges related to sustainability and environmental complications [1] have drawn the attention worldwide towards sustainable energy resources [2]; plant based biofuels, though, have presented great potential in this regard, but have also raised concerns about food to fuel competition for crops along with competition for arable land and water resources. Therefore, the recent biofuel paradigm shift is towards using more sustainable biofuel production options such as utilizing oleaginous microbes, algae and other such resources [3]. Oleaginous microbes have the inherent property of storing >20% lipids in their dry cell biomass under nitrogen deficient conditions. Additionally, these microorganisms have also shown great potential as the fatty acid profile can be changed through alterations in the substrate [4]. The primary components of fatty acids generated by oleaginous bacteria have 16 or 18 carbons, similar to vegetable oils such as rapeseed and soybean [5]. However, due to the high cost of cultivation substrate, which represents 40–80% of total biodiesel production costs, the economic viability

of microbial oils remains questionable [6]. Therefore, in order to achieve cost-effective and long-term microbial oil production, oleaginous microorganisms should be grown on low-cost substrates.

The only limitation that has been identified is that sugar is usually employed for the production of lipids, which considerably raises the production cost. Luckily, a vast variety of these oleaginous microbes can use waste as substrate for the biofuel production [7–9]. For example, it was reported by Fontanille et al. [10], that volatile fatty acids can be volarized by oleaginous yeast *Yarrowia lipolytica* strain MUCL28848 for the bio production of microbial oils. In another study, Gong et al. [11], indicated the potential of *Cryptococcus curvatus* ATCC20509 for producing microbial lipids by using acetic acid waste. A huge amount of food waste is generated on a daily basis from domestic and food industrial processes. According to the reported data, one-third of all produced food is wasted, amounting to about 1.3 billion tons per year. Fruit and vegetable waste accounts for the greatest share of food waste, contributing 45% of total production loss [12,13].

Previously, the conversion of waste to the bioproducts have remained an area of great promotion and conversion of waste particularly municipal waste to the value-added products. Among these, waste conversion to energy is not a novel technology, but is limited to mere application of waste incineration plants and biogas production on a larger scale. Among these, the potential of wastewater based biorefinery is a relatively novel idea and, therefore, extensive work is required to capture the true potential of wastewater based bioprocesses. The use of waste-based biorefinery is not only restricted to the recovery of bioenergy and biopolymers, but also provides a wonderful potential for better value stream management. Along with these advantages, the microbial oil and bio-products produced a typically non-toxic ecofriendly product [13]. Therefore, the application of the waste based biorefinery concept offers a greener and sustainable production option and can play a substantial role in alleviating the global environmental stresses [14–18]. The waste biorefinery model not only ensures environmental benefits, but also provides economic benefits. Since the concept uses the application of reuse, recycle, rebuild, the overall concept adopts the circular economy concept rather than a liner economy model of make, take and dispose [19].

Identifying the significance of the waste treatment and its potential in the bio-energy production through oleaginous microbes, requires defined monitoring of the system performance. The objectives of the present study were to identify the potential oleaginous bacteria having the capability to accumulate lipids using fruit waste as sole substrate. In this regard, the present study was conducted where three novel bacteria were isolated from crude oil samples and applied to different types of fruit waste in the aerobic treatment process. Further, a circular economic model is presented to highlight the potential of work in the subject field application.

2. Materials and Methods

2.1. Preparation of Bacterial Culture and Identification

Previously isolated strains (AF3, KM1, and KM10) from crude oil were recovered from the stock and were enriched using nutrient broth medium [3]. The culture conditions were 30 °C for 48 h at 200 rpm until a maximum OD 600 nm of 1.0 was achieved. The best oleaginous strains KM1 and KM10 were identified using 16S RNA gene assay.

2.2. Preparation of Feed Stock

In the present study, three types of fruit waste samples were collected from the fruit and vegetable market located in Islamabad city. These were apple waste, mango waste and orange waste. Mango and oranges are season fruits; therefore, there samples were collected in the respective season and stored in the low temperature storing unit at 0 °C prior to experimentation. To conduct a waste treatment experiment, slurries were created by blending each waste with water in a 1:19 (w/v) ratio. Each waste slurry was assessed for

physicochemical properties such as electrical conductivity (EC), pH, total dissolved solids (TDS), total solids (TS), VS and Chemical Oxygen demand (COD).

2.3. Experimental

Initially, the inoculums were prepared using 1 L culture flask for each strain and inoculated with the respective culture strains (OD = 1.0) at 1 mL/L. To conduct a waste treatment experiment, slurries were prepared by mixing each waste with water in a 1:19 (*w*/*v*) ratio. The shake flask experiment was conducted in a 250 mL flasks with working volume of 200 mL and head space of 50 mL was left for mixing. Each waste slurry sample was inoculated (inoculation loading 50 mL/1000 mL of the waste slurry) with each of the respective bacterial strain (AF3, KM1, and KM10) (Table 1). The experimental units were incubated at 30 °C in a shaking incubator at 150 rpm, where the periodic samples were taken for every 24 h. All the experiments were conducted under aerobic conditions at 30 °C temperature in the incubator with continuous mixing at 150 rpm. The detail of experimental layout is described in Table 1.

Table 1. Experimental Layout and conditions.

		Oleaginous Bacterial Strains			Experimental Conditions
		AF3	**KM1**	**KM10**	
Waste Types	Apple waste	AW-AF3	AW-KM1	AW-KM10	• Batch capacity = 250 mL • Working volume = 200 mL • pH = 7 • Temperature = 30 °C • Duration = 0–168 h • Shaking = 150 rpm
	Mango waste	MW-AF3	MW-KM7	MW-KM10	
	Orange waste	OW-AF3	OW-KM1	OW-KM10	

2.4. Effect of Bacterial Strains on Physico-Chemical Characteristics of Waste

The effect of isolated oleaginous bacterial strains on different waste types were studied for the duration of 168 h. The waste samples were periodically monitored for change in pH, EC and TDS content using multi meter MM400+. The significant waste degradation indicators VS and COD were also monitored for 96 h simultaneously with lipid accumulation estimation.

2.5. Circular Economy Model for Waste Degradation

Waste degradation and biorefinery concept were put forth in a circular economy model with a few modifications. The waste management elements were given as the cumulative effect of waste collection, separation and maintenance. Overall, the cost effectiveness of system operation was provided as net production cost of three tiers where installation cost was replacing maintenance cost of the system in second and third tier. The cost effectiveness, thus, is a factor of payback value of the operating system for 1 L substrate volume. A linear regression equation was extended to predict the cost effectiveness of the system with controlled conditions for scale up production of 1000 L.

All the values are presented in PKR. Labor cost is calculated as per Federal labor cost of 800 PKR/day. The electricity cost is calculated as per ISESCO cost for commercial use. Establishment cost includes the cost of aerators, water tankers, construction and installation for 1000 L water treatment unit. The Gross Biolipids production from the maximum output of current culture conditions is 240 mL/1000 L of the wastewater (2.4 L for the proposed pilot scale setup). Indirect cost of waste management indicates the treatment cost of 500,000 PKR/1000 L WW/h.

2.6. Analysis

2.6.1. Lipid Accumulation and CDW

Lipid extraction form the biomass was performed using Bligh and Dyer [20] chloroform methanol extraction method. The oven dried (at 65 °C) biomass was used for the

extraction process and the lipid content was calculated gravimetrically. For the estimation of lipid content, the selected isolates were grown on the waste as decried in the previous section. Subsequently, at the stationary phase growth, the cells were harvested by centrifugation for 15 min at 5000 rpm. The cell biomass was washed twice with deionized distilled water to remove excess nutrients [21]. The cell pellets were dried for 24 h at 60 °C to constant dry weight. Finally, the CDW was measured and further analyzed for lipid accumulation capacity by the gravimetric method [22]. The CDW of the biomass produced was estimated gravimetrically.

2.6.2. VS and COD

The VS content of the waste samples was estimated as the ignition weight loss at 550 °C of the dried samples, using formula as used by Qadeer et al. [3]. Open reflux method was used for the estimation of COD in the waste samples as presented in APHA, 2005 [23].

2.6.3. Identification of Bacterial Strains

The 16S rRNA gene assay was performed to identify the most effective bacterial strains, KM1 and KM10. PCR was used to amplify the 16S rRNA gene using specific primers. Montage PCR clean up kit was used to purify PCR products, and Big Dye terminator cycle sequencing kit was used for sequencing. An Applied Bio systems 730XL automated DNA sequencing device was used to resolve sequencing products. The phylogenetic tree was constructed using closely related strain sequences taken from the NCBI database using BLAST searches for bacteria. The strains KM1 and KM10 were found to be closely related (99% similarity) to the previously known species *Serratia surfactantfaciens* and *Serratia liquefaciens*, respectively.

2.6.4. Data Analysis

The data were subjected to descriptive statistical analysis test for calculation of mean and maximum values, where regression analysis was conducted using Minitab software version (17.01). The 3-D surface graphical plots were used to highlight the association of lipid accumulation with different factors.

3. Results

3.1. Removal of Organic Matter from the Waste Slurry

The removal of organic matter from the waste slurry was mentioned as a factor of volatile solid removal (%) and COD removal from the waste samples. Figure 1A–C clearly indicates that there is a sharp decline in the VS % of the waste samples due to inoculation. Of the initial VS, 40–60% was removed by the bacterial strains in different wastes. The highest removal efficiency was observed for KM10 in AW followed by OW, that is, >60% and >50% of the initial VS was removed during the course of incubation of 168 h (Figure 1C). The removal percentages indicated the potential of these bacterial isolates in reducing the overall pollution load of fruit waste containing wastewater. Similarly, Figure 2A–C represents a strong potential of the bacterial isolates for the removal of COD from the waste slurry which is concurrent to VS removal indicated in Figure 1. The COD removal by all the three strains remained around 40–50%. Comparatively, the strain AF3 gave slightly better results than other two strains representing 35–38% removal of initial COD after 168 h of incubation.

3.2. Lipid Accumulation by the Bacterial Cells

The interactive effect of lipid accumulation with VS and COD is presented in Figure 3A–F. The 3-D surface plots used indicated there is a strong relationship between the lipid accumulation and the time duration, but once the peak is achieved at 96 h, the lipid accumulation and time duration demonstrate an inverse relation. The stronger association can be observed between the lipid accumulation and the COD removal and lipid accumulation and the VS removal from the systems. The maximum lipid accumulation achieved by the strain

AF3 was up to 21% in MW and OW at 96 h of incubation. A steady rise in COD in all the waste samples, namely, AW, MW and OW, were clearly indicted in the surface plots. The depression after 96 h of incubation indicate that the maximum lipid accumulation can be achieved at 96 h of incubation. In Figure 3E a smoothed dome shaped surface plot represents a steady association of lipid accumulation with VS removal from the MW by AF3.

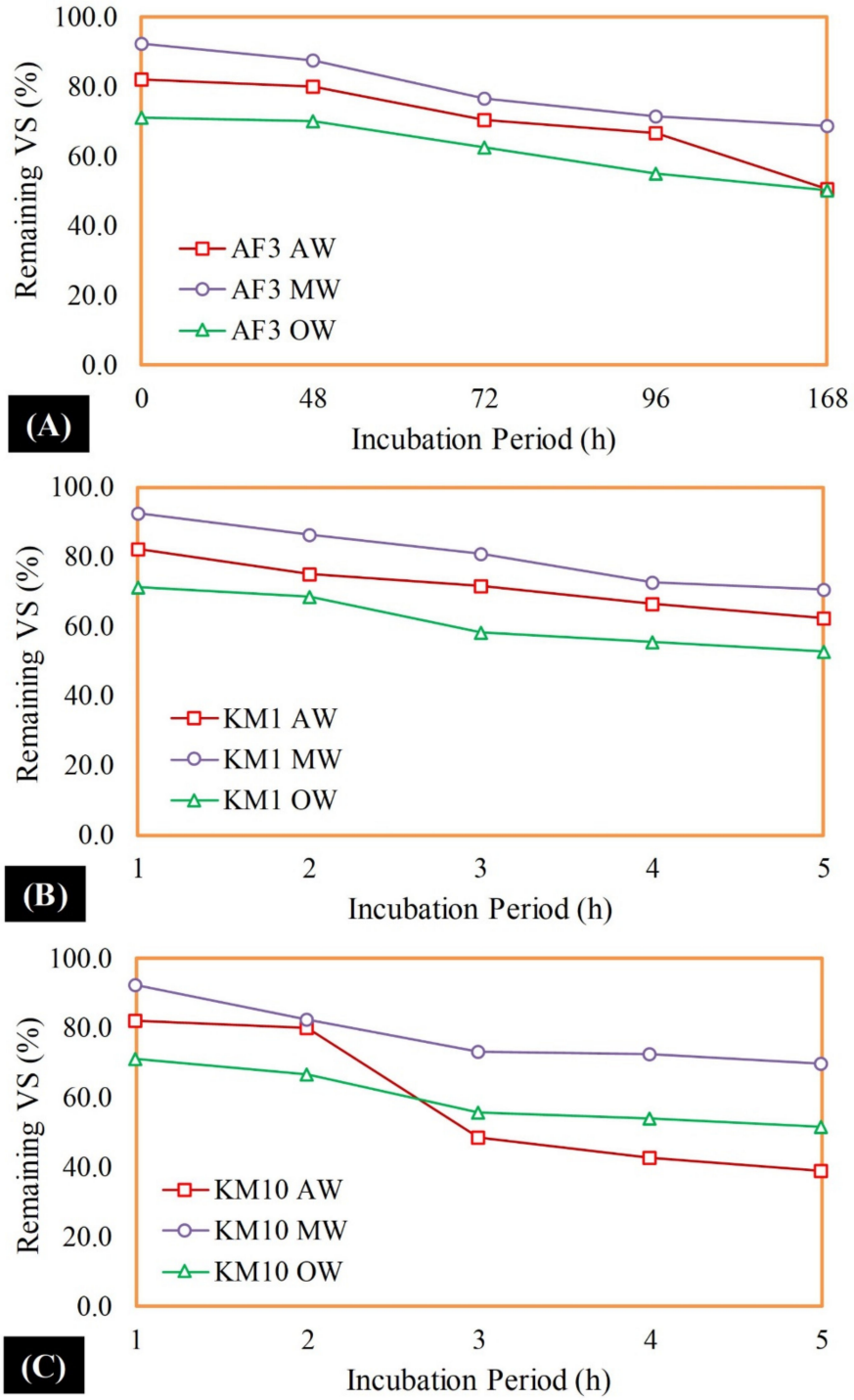


Figure 1. VS removal from the different waste types during aerobic biodegradation of the waste slurry by different bacterial strains; (**A**) AF3 strain, (**B**) KM1 strain, (**C**) KM10 strain.

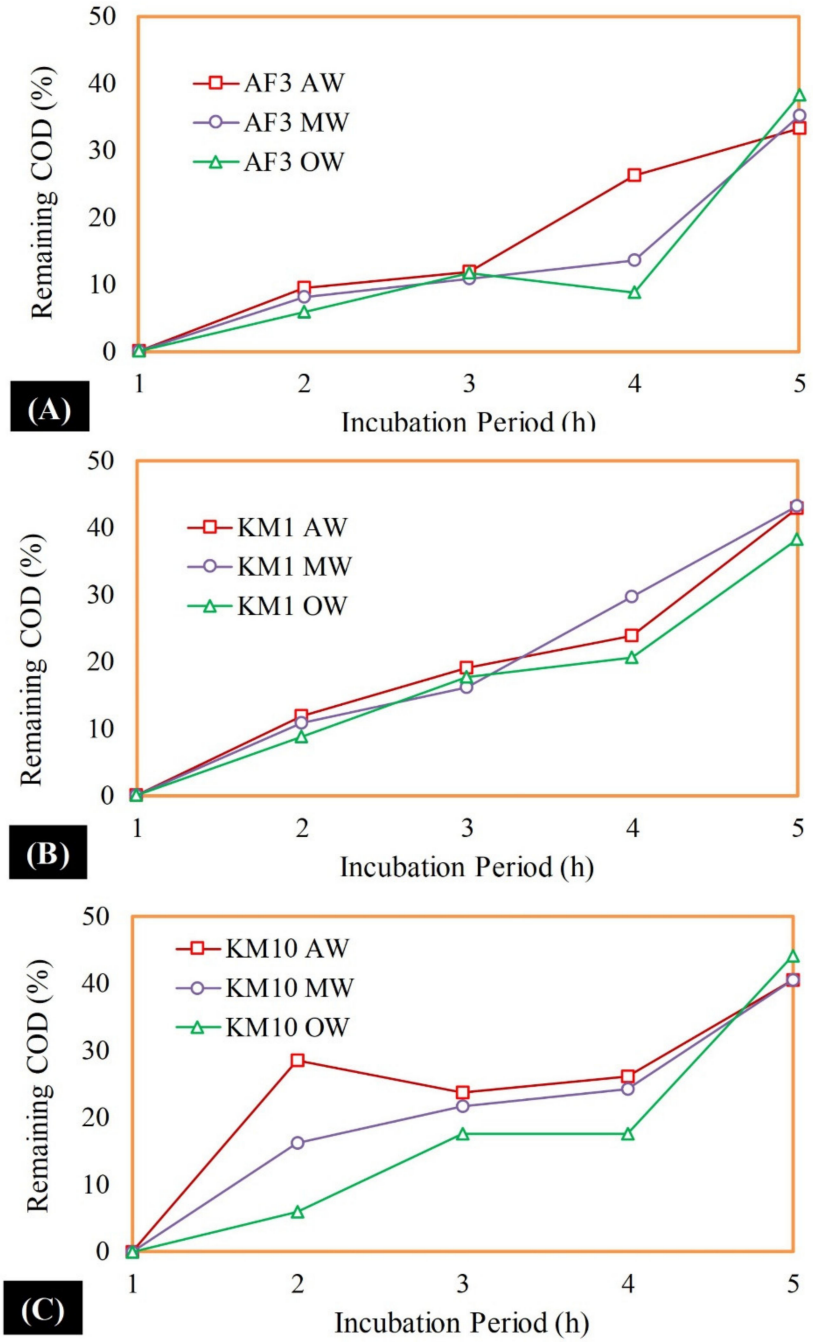


Figure 2. COD (mg O_2/L) removal from different waste types during aerobic biodegradation of the waste slurry by different bacterial strains; (**A**) AF3 strain, (**B**) KM1 strain, (**C**) KM10 strain.

Similarly, the potential of KM1 for lipid accumulation with simultaneous waste degradation and their interrelationship is presented in Figure 4A–F. Lipid accumulation (25.4%) was maximum in KM1 using MW as substrate (Figure 4D). The interaction plots again represented a strong association of lipid accumulation with the process duration; however, this performance is limited up to 96 h only afterwards the lipid accumulation started to decline. This could be clearly observed by the depression at 96 h of incubation of surface plots (Figure 4A,C). The accumulation of the lipids can also be found strongly related to the organic matter removal efficiency.

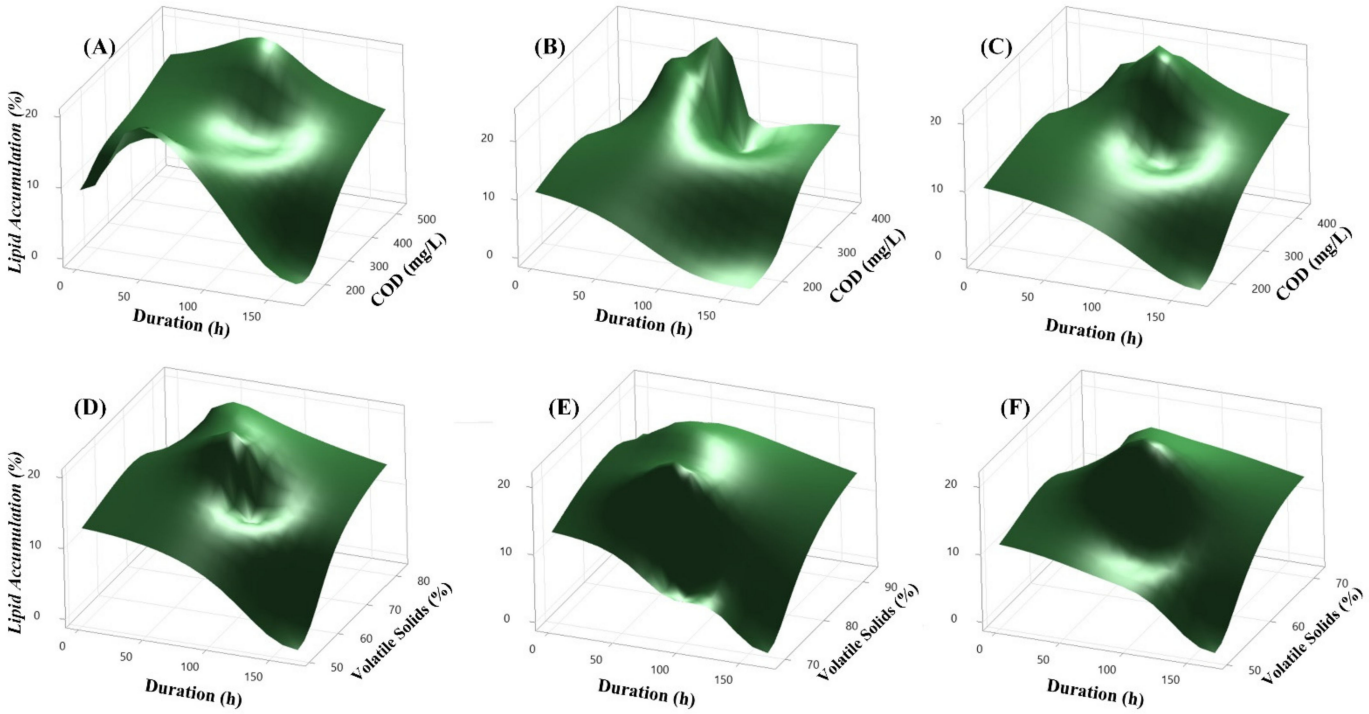


Figure 3. Surface Plot of Lipid Accumulation by bacterial isolate AF3 in different types of waste vs. COD and Volatile solids: (**A**) COD in AW, (**B**) COD in MW, (**C**) COD in OW, (**D**) Volatile solids in AW, (**E**) Volatile solids in MW and (**F**) Volatile solids in OW.

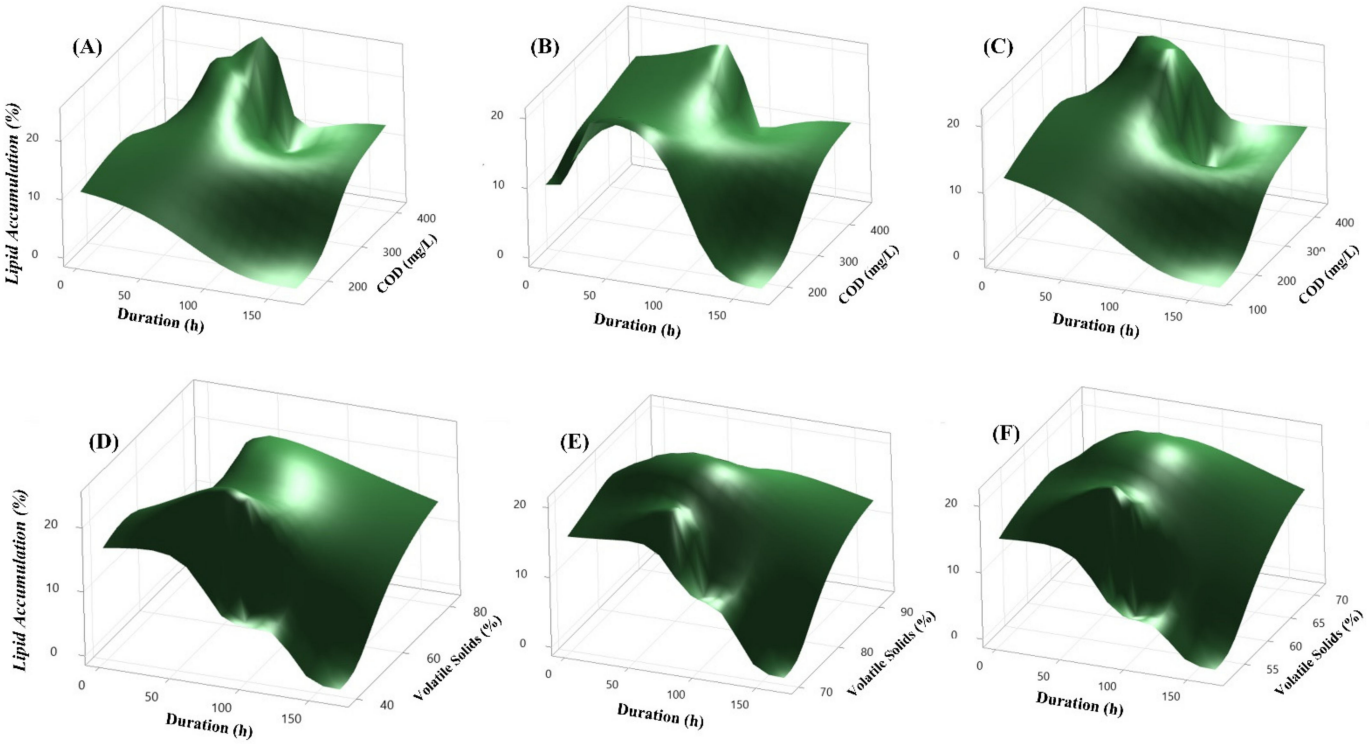


Figure 4. Surface Plot of Lipid Accumulation by bacterial isolate KM1 in different types of waste vs. COD and Volatile solids: (**A**) COD in AW, (**B**) COD in MW, (**C**) COD in OW, (**D**) Volatile solids in AW, (**E**) Volatile solids in MW and (**F**) Volatile solids in OW.

A similar effect can also be observed from Figure 5A–F. The maximum lipid accumulation (24.2%) observed by the inoculation of strain KM10 was observed in AW (Figure 5A).

This accumulation is parallel to the maximum COD change in the waste samples that decreased by26 units in 24 h. At the same time point, the substantial VS change can also be observed (Figure 5B). So over all the interaction plots indicated a substantial link between the observed parameters of organic matter removal.

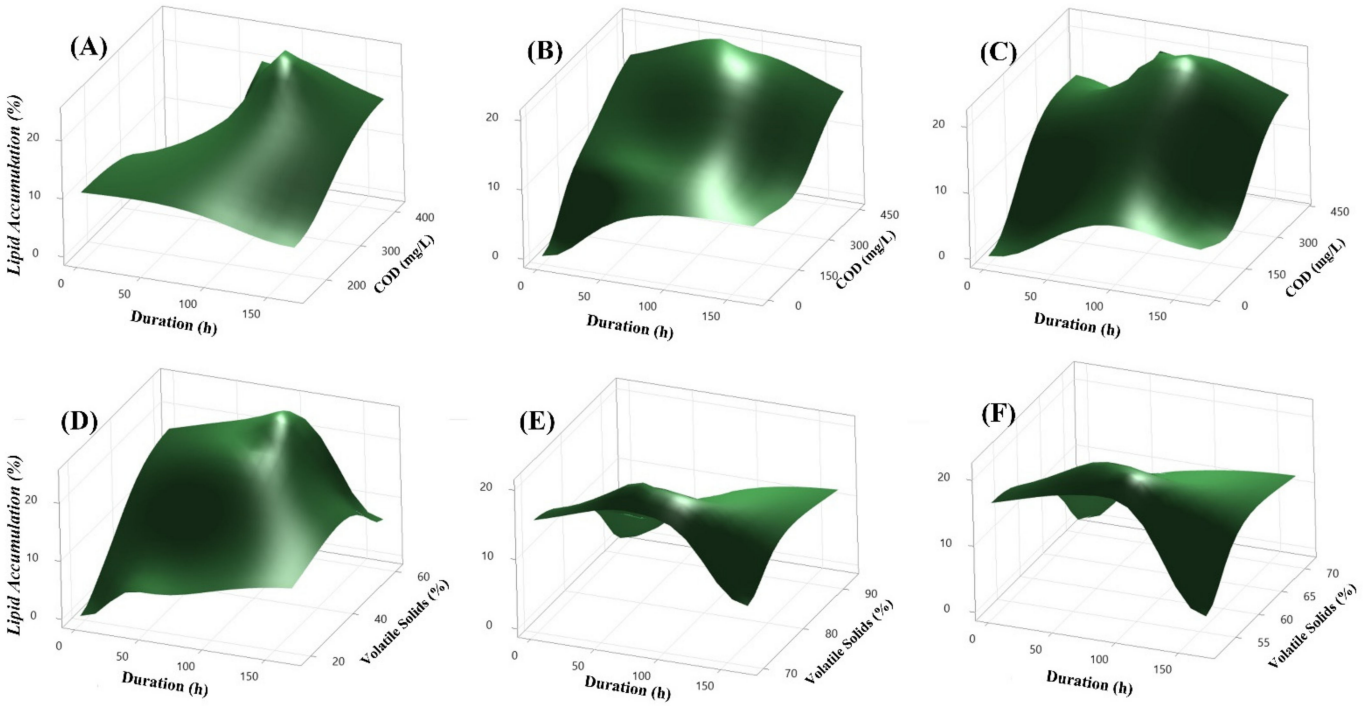


Figure 5. Surface Plot of Lipid Accumulation by bacterial isolate KM10 in different types of waste vs. COD and Volatile solids: (**A**) COD in AW, (**B**) COD in MW, (**C**) COD in OW, (**D**) Volatile solids in AW, (**E**) Volatile solids in MW and (**F**) Volatile solids in OW.

3.3. Bacterial Cultivation Profiles

The effect of inoculation on the physicochemical characteristics of the waste slurry is presented in Figure 6. The change in pH indicated a steady rise in pH of all the waste samples irrespective of the waste type and inoculant provided. However, in OW inoculated with AF3 there was a slight decline in pH at 72 h of incubation. Interestingly, in all the experimental units, the pH remained between 6 and 8; additionally, at 96 h of incubation, a slight decrease in pH was observed for all the inoculated samples. Figure 6b clearly indicates the variations in EC values in the experimental units. Overall, the change remained non-significant in terms of EC variation. Similar to the variations observed in pH values, a slight lowering in EC values has been observed after 96 h. This drop was more obvious for OW by the strain KM1 and KM10; however, no such drop was observed by AF3. Another interesting observation was the drop in EC by the bacterial inoculation in MW was only observed by the strain KM10. A consistent rise in TDS has been observed in most of the experimental units. For instance, inoculation with AF3 resulted in approximately 60 units rise in the TDS (g/L) of the OW samples; however, the TDS started to decline afterwards. Similarly, inoculation with KM1 resulted in more than 60, 70 and 65 units rise in AW, MW and OW, respectively, until 96 h of incubation, followed by a non-significant decline afterwards.

3.4. Waste to Energy: A Circular Economy Model

The waste to energy circular economic perspective for the current study is presented in Figure 7. The critical analysis of the process design and operation indicated that one-time establishment cost is the major capital cost of the process were running tiers of the small scale 1000 L treatment plant can be eliminated in follow-up tiers. The overall

cost, 561,457 PKR, can, overall, be greatly reduced by including indirect cost of waste management. Similarly, the fuel remuneration can also play substantial role in reducing the overall cost with a system capacity of producing 2.4 L of bio lipids per tier. The overall system efficiency, though, could be enhanced by using high lipid accumulating strains or optimizing the maximum lipid accumulation.

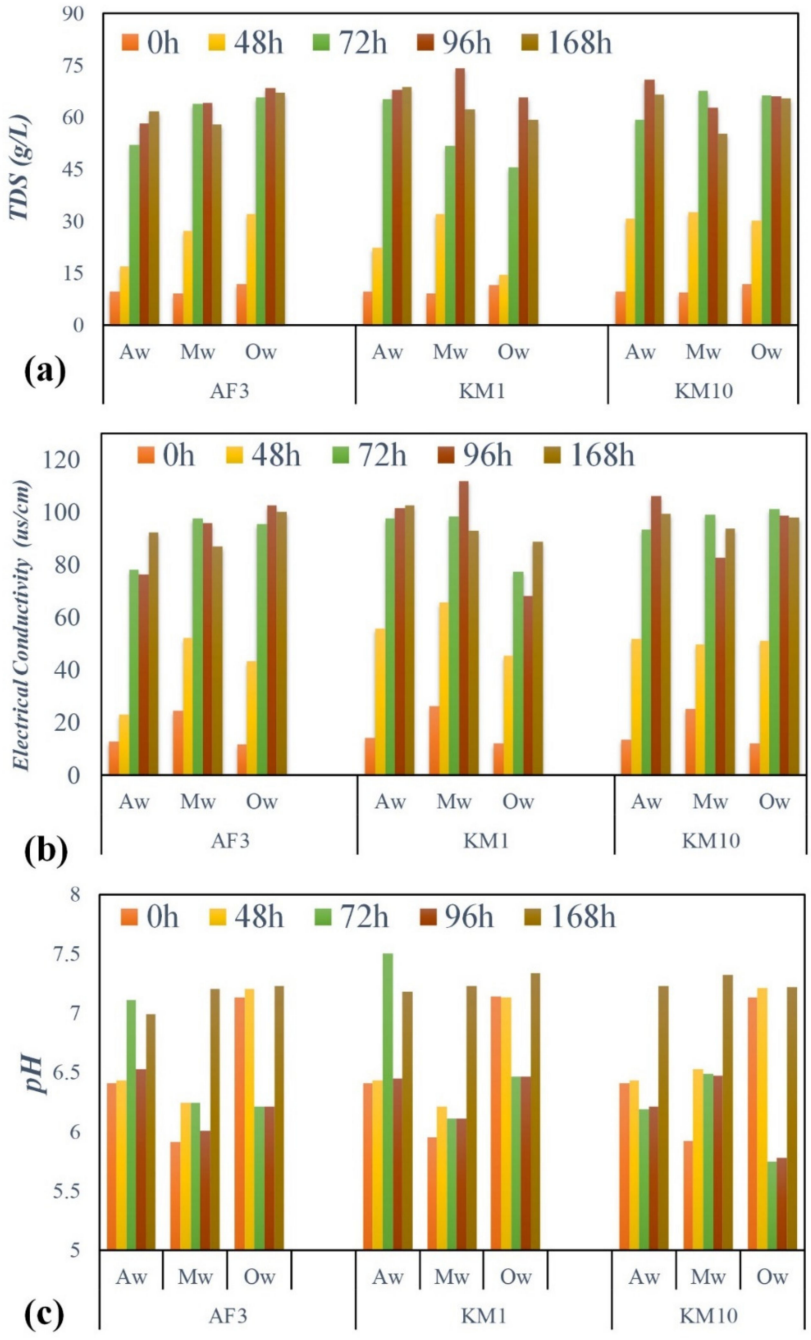
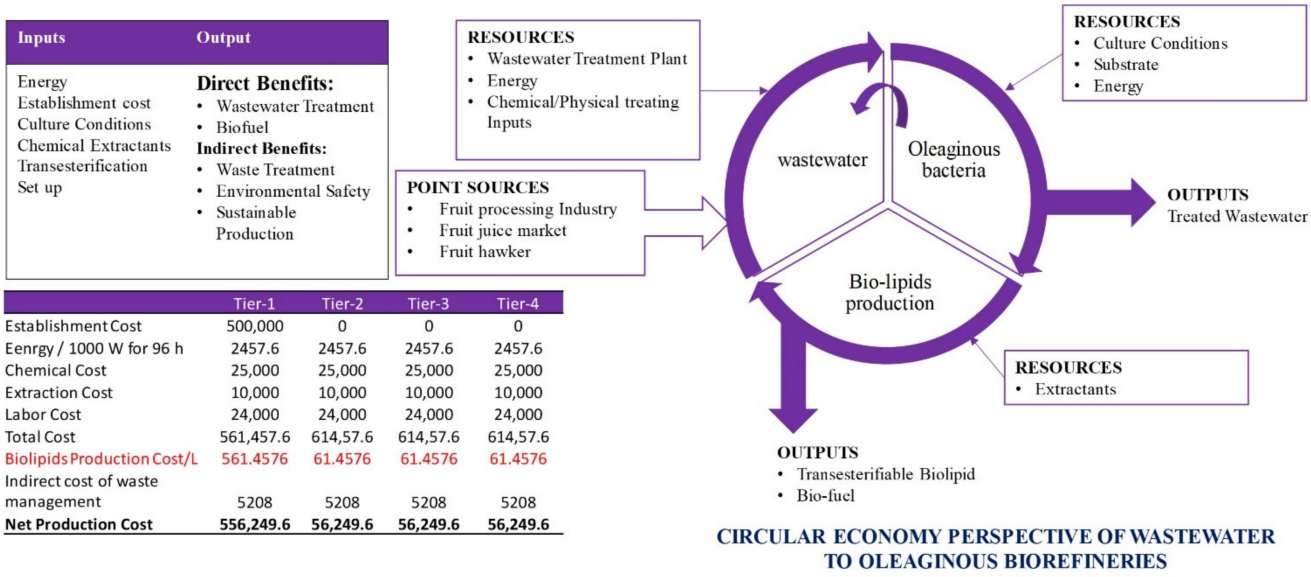


Figure 6. Variation in Total Dissolved Solids (**a**), Electrical conductivity (**b**) and pH (**c**) of the waste samples observed during aerobic biodegradation of the waste slurry by different bacterial strains.



	Tier-1	Tier-2	Tier-3	Tier-4
Establishment Cost	500,000	0	0	0
Eenrgy / 1000 W for 96 h	2457.6	2457.6	2457.6	2457.6
Chemical Cost	25,000	25,000	25,000	25,000
Extraction Cost	10,000	10,000	10,000	10,000
Labor Cost	24,000	24,000	24,000	24,000
Total Cost	561,457.6	614,57.6	614,57.6	614,57.6
Biolipids Production Cost/L	561.4576	61.4576	61.4576	61.4576
Indirect cost of waste management	5208	5208	5208	5208
Net Production Cost	**556,249.6**	**56,249.6**	**56,249.6**	**56,249.6**

Figure 7. Waste to energy circular economy model using oleaginous bacteria under different tiers of capital and output costs.

4. Discussion

4.1. Concurrent Lipid Accumulation and Waste Degradation

The waste degradation and concurrent lipid accumulation is clearly visible in the current study. It was observed that the waste degradation measured as a factor of VS removal and COD removal is paralleled by the lipid accumulation. In most of the bacterial strain, the maximum lipid accumulation was obtained up to 96 h with increased waste degradation potential; however, with further increase in time waste, degradation also increased with slight reduction in lipid accumulation. The maximum waste degradation was obtained as the maximum lipid has been accumulated with the respective bacteria strains. However, if the incubation time is further extended, the further waste degradation can be achieved. This can be attributed to the fact that oleaginous bacteria actively accumulate lipids under carbon surplus conditions and, thus, to receive maximum carbon from the waste these bacteria actively degrade the waste as a side benefit. The accumulation of lipids by oleaginous bacteria and parallel COD removal has also been observed previously by Goswami et al. [24], where 64% COD removal was achieved in raw biomass gasification wastewater using oleaginous strain *R. opacus*. In the current study, the maximum COD removal was achieved by the strain KM1 in all three types of waste (>50%); comparatively lower COD removal was achieved by AF3 and KM10 but the removal percentage was still more than 40% in 168 h. Interestingly, the strain KM9 removed approximately 59% of COD in 96 h. *R. opacus* is reported in many studies for COD removal and simultaneous lipid accumulation [25,26], where, in other studies, *Gordonia* sp. have also been reported for efficient lipid accumulation with waste treatment.

Previous studies have also indicated that oleaginous microorganisms can accumulate an impressive amount of lipids using waste, either directly or in modified form [27]. Several agro-industrial substrates, such as wheat straw, wheat bran, rice straw, rice husk, corn stalk, corn cob and various types of fruit and vegetable waste, have established their competence as substrates for oleaginous biorefineries [28]. The accumulation of the lipids was observed more prominently in hydrophilic wastes [29]. Particularly, bacterial isolates have shown significant strength to transform wheat bran, fruit and vegetable waste, food processing waste, molasses and potato waste into bacterial biolipids. For instance, bacterial isolates *Gordonia* sp. and *Rhodococcus opacus* PD630 stored 96% and 93% of lipids, respectively, using sugarcane molasses as substrate; competitive results from orange waste and carob waste were also obtained from the same isolates when grown, respectively [30]. Interestingly, in the current study, it was observed that maximum lipid accumulation by AF3 was achieved

in orange waste, though the accumulation rate was lower compared to the previous studies (21%) with >40% of VS removal. While KM1 stored the most lipids in MW, KM10 stored the most in AW. This difference in lipid accumulation behavior can be explained by the inherent property of each strain to store a different amount of lipids under different substrate conditions, where it is reported that the lipid accumulation is dependent on both the oleaginous specie and culture conditions [31]. That is why the bacterial lipids are encouraged; the substrate modification and associated lipid accumulation characteristics of the oleaginous bacteria can be easily modified as per requirement. Additionally, a variety of substrates can be exploited for lipid production [32]; one of such example is the use of *R. opacus* for lipid accumulation using waste office paper hydrolysate [33].

4.2. Effect on Physic-Chemical Characteristics of Waste

The physic-chemical characteristics of the culture conditions strongly effect the lipid accumulation characteristic of the bacterial strains. Similarly, the change in pH, EC and TDS can be taken as a factor to monitor the waste degradation efficacy of the bacterial strains. In the current study, without any adjustment, the pH of the biodegradation experiments remained close to neutral range, i.e., 6 to 8. However, a steady decline in pH by AF3 at 96 h in MW, at 72 h in OW; by KM1 at 72 h in OW and by KM10 at 72–96 h in OW indicated the possibility of production of extracellular fatty acids that can be linked to the higher lipid accumulation at the respective points (Figure 1). Where at the similar point of incubation a higher EC indicates active solubilization of waste for the provision of sufficient nutrition to the actively growing and accumulation bacterial isolates (Figure 6). It was also reported that the increase in EC of degradation experiment can be taken as in indicator of loss of organic matter from the waste samples [34] and release of mineral salts from the waste [35]. Therefore, the increase in EC is attributable to the mineralization of organic matter in the aerobic experiment [36], which can be observed by the loss of VS and COD removal from the waste samples during the course of incubation. This concept is further confirmed by the increase in TDS of the aerobic biodegradation samples (Figure 6). The increase in the TDS content at some points of the experiment represents the solubilization of the undissolved inorganics in the waste, whereas a regular decrease could be related to the use of solids solubilized by the activity of the bacterial strains [37]. This further explains that the bacterial isolates tend to solubilize the waste components, which can be a potential source for the lipid accumulation.

4.3. Economic Feasibility of the Food Waste Slurry to Bacterial Biorefinery

The overall biolipids production cost estimated in the current experimental setup with wastewater to oleaginous biorefinery perspective is 62 PKR/L in the second tier and onward, which is far less than the fossil fuel price, which is around 160 PKR/L (1.21 USD maximum). Different studies have indicated that a low-cost substrate can greatly reduce the production cost of the oleaginous microbe-based biofuels [38,39]. Similarly, the studies have also indicated that microbial lipid production is economically feasible technology [8]. The use of circular economy over linear economy provides multiple benefits as per linear production perspective; the following could be the possible probabilities.

Wastewater → Wastewater Treatment Plant → Treated Wastewater × Bioproducts (only input cost applies)

Substrate → Oleaginous bacteria → Extraction → Esterification → Bio-fuel (high input cost)

The circular economy perspective presented in Figure 7 combines and synchronize both processes where the treatment of wastewater and its associated cost can be fully avoided by incorporating the oleaginous bacteria. For instance, Chintagunta et al. [40] has presented a great economic potential of integrated biofuel production systems. The circular economic perspective is also discussed as a potent model for economic benefits for Brewer's spent grains [41]. The economics research has also indicated that the use of circular economic model over linear economic model has edge to attain the sustainability [42,43].

Therefore, altering from linear to circular economy can significantly enhance the system performance and open a new smart system of biofuel development form wastewater treatment plants.

5. Conclusions

The bacterial strain KM10 obtained the highest lipid accumulation (>25%) using AP as a substrate, followed by the KM1 (>24%) using MW as a substrate. The lipid accumulation matched with organic matter degradation where >40% removal of VS was achieved; therefore, the process could be used as a potential waste treatment option. The 16s RNA gene sequencing results showed that these KM1 and KM10 strains were identified as *Serratia surfactantfaciens* and *Serratia liquefaciens.* In terms of the circular economy, the system has a high economic value since wastewater treatment and its accompanying costs may be completely eliminated by using oleaginous bacteria. The transition from a linear to a circular economy can considerably improve system efficiency and open the door to a new smart biofuel production system from waste. Overall, the use of organic-based biorefinery concepts offers a greener and more sustainable production alternative, and may play a significant part in reducing global environmental strains.

Author Contributions: Conceptualization, S.Q. and A.K.; methodology, S.Q.; software, S.Q.; validation, M.A., S.M. and A.R.S.; formal analysis, S.Q.; investigation, S.Q. and M.A.; resources, S.Q.; data curation, S.Q.; writing—original draft preparation, A.K.; writing—review and editing, A.K.; supervision, A.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. However, the work was conducted and supported by Environmental Sciences Lab. at Department of Environmental Sciences, PMAS Arid Agriculture University.

Data Availability Statement: The data will be available from the corresponding author upon a reasonable request.

Acknowledgments: The authors acknowledged Department of Environmental Sciences, PMAS Arid Agriculture University for all technical and material support during execution of this work.

Conflicts of Interest: The authors declare no financial and personal conflict of interest.

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