Trans-Esterifiable Bio Lipids Synthesis in Bacterial Isolates Using Limonene Containing Waste

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Abstract

Bio-oil is recognized as a sustainable eco-friendly source of energy and a potential candidate to substitute the use of petro-diesel. In the present study, cultivation of a novel oleaginous culture KM9 was evaluated for bio-oil production potential and compared with a known oleaginous specie *R. erythropolis*. These strains were assessed for biomass production and growth was optimized for their respective nutrient requirements and physiochemical conditions. The cultivation of strains was also evaluated for their potential to use orange waste as substrate, lipid accumulation and simultaneous waste minimization. The results showed that KM9 reduced more than 50% of organic matters from the waste (47% VS removal and 60% COD removal) and achieved a maximum lipid accumulation of up to 38% of cell dry weight (CDW). In contrast, *R. erythropolis* stored a maximum of 27% lipids in the cell biomass. Florescence microscopy confirmed the accumulation of lipids by both strains. GC-MS of the trans esterified lipids revealed that both strains tended to store more saturated fatty acids (SFA) (38.39% by KM9 and 39% by *R. erythropolis*) when using limonene modified media. However, the use of orange waste triggered the accumulation of mono unsaturated fatty acids (MUFA). The high proportion of palmitic acid and stearic acid indicated its close resemblance to the plant based bio-oils. This study gave insight about bacterial lipid accumulation in the novel isolate KM9 for bio-oil production, which also provided additional benefit of waste minimization. Therefore, the oleaginous KM9 can be used to produce microbial lipids as a potential alternative oil from orange wastes, providing a novel process for sustainable production of biodiesel to meet escalating energy demands and waste minimization and utilization into value added products.

Key Points

- The new bacterial strain KM9 represented high waste removal of an antimicrobial agent Limonene
- The study indicated more than 50% removal of COD with 38% lipid accumulation in the CDW
- The GC-Ms Analysis confirmed the accumulation of transesterifible waste in the bacterial cell

1 Introduction

Management of citrus waste is a subject of great importance worldwide due to its high production rate. Among various citrus corps, the waste produced from oranges either during process or storage is getting serious concern of scientific community, both in terms of its management and resource recovery. Global orange production rate is forecast up to 2.4 million metric tons in the year 2016-17 [1], where FAO estimates puts one third of the fruits and vegetables in the waste category. Other estimates placed that 50% of the processed fruit weight is transformed in to waste containing peels and membrane residues along with juices [2, 3]. Thus, high production rate is usually paralleled with the high volumes of waste generation. Previously, specifically orange waste was utilized for development of value added products [2], such as pharmaceutically important compounds [4], pectin [5, 6] enzyme production [7] and orange

beads for wastewater treatment [8]. A little potential of orange peel waste has also been identified in biofuel industry using C*hlorella vulgaris* [9]. However, most of these processes include only orange peel and do not consider the management of waste pulp or liquid. The other limitations are associated with their extraction cost and requirements of pretreatments that demands energy and chemicals. Therefore, the ultimate fate of orange waste is usually landfills [10]. One of the viable options for the treatment of waste could be anaerobic digestion but is hindered by the presence of an antimicrobial agent 'limonene' [11]. Therefore, there is dire need of time to find ways to degrade citrus waste with particular focus on limonene. The rise in environmental awareness and waste to energy conversion has broadened the concept of waste utilization for microbial lipid production.

In past few years the utilization of microbial lipids for the oleochemical synthesis has attracted much attention [12–15]. Particularly the synthesis of fatty acid methyl esters (FAMEs) has gained substantial progress for their applications in biodiesel production [13] (Meng et al., 2009). Therefore, advancements in microbial lipid based FAMEs production focused on identifying key factors associated with lipid accumulation [16–19]. Diverse types of micro-organism are known for their bio-oils potential which include microalgae, yeasts, molds and bacteria. Certain algae are reported which can store lipid content up to 70% of lipid in general and under certain conditions up to 90% of lipids of dry biomass [20]. Similarly, some fungal specie e.g. *Crptococcus curvatuscan, Rhodosporidium sp*. and *Rhodotorula sp.* have potential of lipid storage up to 60% [13]. Some bacterial isolates like *R. erythropolis, Rhodococcus opacus, Streptomyces sp*., *Acienetobacter calcoaceticus, Arthrobacter sp., Nocardia sp., Mycobacterium sp., Bacillus alcalophillus* [13, 16], also reported for bio-lipid potential. Although bacteria have relatively low potential of lipid storage compared to fungi and algae still it can be competent producer due to their high growth rate. Therefore, present study is aimed to evaluate the potential of bacterial culture KM9 for waste treatment and also it's potential in utilizing orange waste as substrate for FAMEs production.

2 Materials And Methods

2.1 Isolation and screening of KM9

Oleaginous bacteria were isolated from crude oil obtained from different oil fields of Pakistan namely Karak MOL, Karak MPCL, Kohat MOL, Attock oil fields and Pindi oil fields. Out of 63 different isolates reported in previous study [21], KM9 also represented high lipid accumulation and biomass production using orange waste as substrate.

2.2 Potential of KM9 cultivation on different types of waste

Initial experiments were conducted to find out the potential of KM9 to degrade different types of waste (apple, mango, orange) and simultaneous lipid accumulation. Waste slurries were prepared by adding 5 g (wet weight) of each solid fruit waste in form of pulp and peels (shredded properly for uniform particle size that is 2-5 mm) to 95 ml of distilled water (DW) to make final volume of 100 ml. All waste solution

was sterilized at 120˚C for 20 minutes using autoclave. Initially the growth of KM9 in the waste was confirmed by measuring the rise in OD_{600} nm of the inoculated waste up to 48 hours using photo electric Colorimeter (ERMA Inc., Tokyo, Japan).

2.3 Potential of KM9 to store lipids using different waste compositions

Different waste compositions were tested to find out the best combination for both growth promotion and percentage lipid accumulation (Table 1). KM9 was introduced in sterilized waste formulations. Aerobic digestion was carried out, under slight agitation (150 rpm) and external temperature of 30°C. For inoculum preparation, culture KM9 was inoculated in LB media and incubated for 48 hours until equaling OD_{600} of 0.7 was obtained. Five ml of this culture was inoculated in each waste formulation of total 150 ml. Sampling was carried out periodically after every 24 hours up to 120 hours from each experiment. The samples were filtered prior to OD_{600} determination to remove any solid particles of the waste, where for determination of lipid accumulation, 5 ml from each culture was centrifuged and the settled particles were used for the lipid analysis using by Bligh and Dyer method [22].

2.3.1 Orange waste degradation study

Waste degradation was monitored as a factor of organic matter degradation in terms of volatile solids and COD (Chemical Oxygen Demand) removal during the aerobic wet digestion process. Sampling was carried out periodically after every 24 hours up till 120 hours of digestion process. Other operating parameters like change in pH, electrical conductivity and total dissolved solids were also carried out

2.4 Potential of KM9 to degrade citrus waste

The culture KM9 was analyzed for its degradation potential of orange waste and compared with *Rhodococcus erythropolis* courtesy obtained from ARS Culture Collection, National Centre for Agriculture Utilization Research USA. Initially *R. erythropolis* was cultured as per supplier's instructions in nutrient broth (NB) media for 48hr at 30°C; the enriched active culture was further maintained at NB-agar slants. For inoculation, each strain was grown in LB media for up to 48 hours at 30°C with slight agitation at 200rpm until OD_{600} was > 0.8. To evaluate the potential of KM9 to degrade the orange waste initial experiment was conducted using 1 ml L^{-1} limonene (a major compound in orange peel waste) supplemented in MSM media.

2.5 Optimization of operational conditions

The operational condition such as pH, temperature and nutrients (C and N) were optimized for cultivation of oleaginous bacteria using limonene containing solution. Initial experiments were conducted to check the effect of nitrogen source and carbon source on biomass production of the bacterial strains using modified MSM media supplemented with 1 ml L^{-1} (v/v) limonene. MSM modified with limonene comprised of 1.0 g L^{-1} each of the NaCl, KH_2PO_4, Na_2HPO_4, 0.5 g L^{-1} $MgSO_4$ and 0.1 g L^{-1} $CaCl_2$, Modified media comprised of three different carbon sources 2 g/L each were used separately that is glucose, sucrose and cellulose using 0.1 g L^{-1} NH_4Cl as nitrogen source in limonene modified MSM. Similarly, three different nitrogen sources $(NH_4)_2SO_4$, NH_4Cl and urea were tested at 0.1 g L^{-1} (w/v) application rate using 1 g L^{-1} (w/v) sucrose in limonene modified MSM. The cultures were incubated at 30 °C and 200 rpm until the OD_{600} started declining after 96 hrs. Biomass production was measured as the estimate of optical density (OD_{600}) using UV-vis Spectrophotometer (Schmadzu UV-1601).

2.6 Lipid visualization using fluorescence microscopy

Intracellular and extra cellular lipids of the bacterial cells were stained using Nile red fluorescence dye (Thermosfisher Scienific). The culture KM9 and *R. erythropolis* were incubated in the MSM modified with limonene and lipid fluorescent images were captured at stationary growth phase to visualize the lipids in the cell biomass. Briefly, 1 ml of cell culture (OD_{600} ≈1) was inoculated with 10 µL of Nile red fluorescent dye (0.033 mg L^{-1}). The cells were incubated for 2 min in dark at 37 °C , [23] (Cea et al., 2015). Nile red fluorescence was measured using Nikon eclipse 80i at fluorescence intensity 570-580 nm and excitation wavelength of 480 nm. The images were captured by Nikon color camera using NIS (Nikon imaging software) elements- advanced research software. The final images were converted to exportable Jpeg files.

2.7 Estimation of lipid accumulation

Two sets of experiments were conducted to evaluate the potential of KM9 to use orange waste and limonene as substrates under optimum conditions. In 1st experiment Limonene was used as model compound to harness potential of KM9 for lipid accumulation. Experiment was conducted in 250 ml Erlenmeyer flasks under batch condition with agitation at 200 rpm at 30°C temperature. The working volume of aerobic digestion unit was 100 ml. Experiment was conducted using 1% limonene with auxiliary carbon and nitrogen source identified from the previous experiment that is sucrose 2 g L^{-1} and 0.2 g L^{-1} NH_4Cl. Each flask was inoculated with equaling proportions of two different types of 3 ml of inoculum of 0.8 OD_{600}. Lipid accumulation % was estimated after 24 hours till the further accumulation is ceased or started declining. The microbial lipids were extracted from the samples were trans-esterified and analyzed through GC-MS.

In second set of experiment, similar inoculation pattern was followed as mentioned above, however, 3 % media as optimized source of carbon and nitrogen (without limonene) was added in the waste slurry and lipid accumulation was measured. As per previous experiment the extracted lipids were trans-esterified and analyzed through GC-MS.

2.8 Analytical methods

2.8.1 Cell dry weight

Cell dry weight was estimated gravimetric method by lyophilizing the pellet obtained in 10 ml of cell culture broth after centrifugation at 3000 rpm for 30 min. The pellet was washed twice with sterile saline solution (1% w/v NaCl).

2.8.2 Lipid accumulation

Lipid accumulation in the CDW was estimated gravimetrically using Bligh and Dyer method [22]. Briefly, the lyophilized biomass (approximately 100-1000 mg) was mixed with 3.75 ml extraction solvent (chloroform: methanol (2:1 v/v) and mixed, followed by addition of 1.25 ml of chloroform. The samples were vortexed for one minute and then 1.25 ml of 1M NaCl was added and vortexed again. Afterwards the working solution was centrifuged at 3000 rpm for 15 min. In the separated phases after centrifugation the upper phase (supernatant) was collected carefully. The lower phase was dried under N_2 stream to get the dried lipids. The lipid % was estimated as measure of gravimetric difference in the CDW.

2.8.3 COD analysis

Chemical oxygen demand (COD) was determined by using closed flux method [24] (APHA, 2005). Samples were digested at 150 ^{o}C in closed reflux vials. The contents of each vial were separately transferred to a 250 ml volumetric flask and then titrated against ferrous ammonium sulphate (FAS) using a ferroin indicator. The COD of the samples was calculated as

$$\mathrm{COD\ (mg\ L-1)} = \frac{(\mathrm{X-A}) * \mathrm{M} * 8000 * 1}{\mathrm{Sample\ volume}}$$

Where; X-A = volume of FAS used for blank- volume of FAS used for volume

M = molarity of the FAS used for the titration.

2.8.4 Trans-esterification of the extracted lipids and determination through GC-MS

Ex-situ trans-esterification of the lipids using acid-methanolysis of the extracted lipids was carried out. The bacteriological lipids were mixed with 1ml f methanol containing 15% H_2SO_4. The methanolysis was carried out at 100°C for 20 min. Afterwards DIW was added to the reaction mixture and then was centrifuged again at 6000 rpm for 15 min (Ryu et al., 2018). The lower organic phase was recovered and analyzed through GC-MS analyzed by GC–MS (Agilent, Santa Clara, CA, USA) using DB-5MS capillary column (30 m 0.25 mm 0.25 lm). 30µ L of $C_{17:0}$ was used as internal standard. Splitless injection mode was used for injecting the sample (at 250° C) using helium (1 mL/min) as a carrier gas as reported earlier [25].

3 Results

3.1 **Potential of KM9 to grow on different types of FW (fruit waste)**

Among three types of fruit waste the growth of KM9 measured as OD_{600} represented that the culture KM9 has highest potential to grow on orange waste as compared to the other two types of waste (Fig. 1). At 24 hours though the highest growth was observed in apple waste but at 48 h highest growth was observed in orange waste. The maximum OD_{600} achieved at 48 hours using waste as sole source of nutrition was up to 0.6. Similarly, in different fruit combinations though the observed biomass production was high in mango and apple combination but lipid accumulation was observed highest in the combinations with orange waste (Fig. 2).

3.2 W**aste degradation potential of KM9**

Potential of KM9 to degrade different types of waste was estimated by organic matter removal efficiency of KM9 in terms of VS (volatile solids) removal and COD removal, the oxidizable organic matter removal is presented in Fig. 3. The remaining COD of initial COD of the waste indicated that there is almost 60% removal of initial COD in all waste types. Where the lowest content of initial COD in orange waste represent that

When tested for different waste compositions at different mixing ratios orange waste still proved to be a better substrate for both growth and lipid accumulation by culture KM9. Each substrate when was used separately it is observed that the though maximum lipid accumulation was achieved by orange waste (Fig. 3). The highest lipid accumulation percentage was achieved using orange waste as substrate that is 38%. Similarly, the substrates having orange waste as a constituent represented better lipid accumulation percentage compared to others waste compositions the (Fig. 3). Though the highest cell growth in terms of CDW (cell dry weight) was achieved by combination of apple waste (25%) and mango waste (75%) but lipid accumulation percentage was even lower than 20%. Individual waste substrate showed highest

growth and lipid accumulation percentage in orange waste, where in case on mango waste though the lipid accumulation rate was higher than apple waste but the growth was very low (0.019 g/L).

Similarly, when estimated for organic matter degradation potential of KM9 as factor of reduction in COD and VS, the highest organic matter degradation was observed in case of orange waste, the corresponding removal of VS and COD in orange waste by culture KM9 was 47% and 60% respectively. Fig. 3 indicates the removal percentage of VS from the waste and remaining COD corresponding to each waste type by culture KM9. Only 37% of total VS were removed in AW and MW, where for OW it was higher. Where in terms of COD removal around 55% removal from initial COD was achieved by culture KM9.

3.3 Optimization for cultivation and biomass production

Since KM9 was found efficient both in terms of lipid storage and waste degradation study therefore optimization study was carried out in comparison with *R. erythropolis.* In temperature optimization studies no significant difference was observed between KM9 and *R. erythropolis.* The maximum growth in both isolates was around 0.7 at 48-72 hours (Fig. 4 a-b). Where after 72 hours of incubation at 30ºC the growth of KM9 and *R. erythropolis* didn't drop drastically. However, at 40ºC the initial growth of KM9 was drastically higher nearly 0.89 which dropped suddenly to 0.68 at 48 hours of incubation and 0.43 at 72 hours of incubation. Similarly, at 50ºC the growth of KM9 was consistently lower as compared to other two temperatures. Likewise, *R. erythropolis* showed higher growth at 30ºC as compared to 40ºC and 50ºC. The optimization of pH at three different pH indicated that pH 6.5 is best for growth of KM9. The maximum growth of both strains was found to be at pH 6.5 (Fig 4 c-d). KM9 represented a better growth rate as compared to *R. erythropolis* the maximum growth was observed 0.7 at 72 hours of interval where a similar growth of 0.68 was observed at 48 hours at pH 6.5 by culture KM9. Where at same pH *R. erythropolis* showed better growth at 48 hours and started to decline after 48 hours of incubation. The consistently low *growth* by both strains was observed at alkaline pH. Therefore, 30ºC and 6.5 pH were better condition to attain maximum growth by both strains.

Similarly, optimization study of carbon and nitrogen sources is presented in figure 5 (a-d). The results revealed that media supplemented with different carbon and nitrogen sources resulted in better growth. Where the maximum g growth was observed for the media supplemented with sucrose as carbon source in both strains i: e OD_{600} up to 0.66 by KM9 and 0.51 by *R. erythropolis*. Where the without additional source of carbon the growth of both strains remained very low (OD_{600} < 0.1) in both strains. The growth increased considerably when additional nitrogen sources were also used with carbon. The growth of KM9 increased at 48 hours and remained considerable good up to 72 h of incubation (fig 5 a-b). Similarly, the growth of *R. erythropolis* also increased with the addition of nitrogen source (fig 5 c-d). Therefore, sucrose and NH_4Cl were selected as best media supplementation for growth of KM9 and *R. erythropolis* in limonene modified media.

3.4 Lipid visualization using fluorescence microscopy

The Lipid accumulation in both KM (9 and *R. erythropolis* during stationary and early growth phase is represented in figure 6. The images clearly represent that *R. erythropolis* showed more lipid accumulation at late stationary phase i: e 72 h as compared to early growth phase. Where KM9 represented lipid accumulation from 48 h and sustained the oleginic property up to 72 h.

3.5 Estimation of lipid accumulation

Lipid accumulation and CDW are presented in Fig. 7. The cell dry weight of KM9 continued to increase up to 72 h of incubation and drops afterwards. The maximum biomass was achieved up to 595 mg L^{-1} of KM9 (Fig. 7a) and 480 mg L^{-1} of *R. erythropolis* (Fig. 7b) at 48 h and 72 h of incubation. The lipid accumulation in the cell biomass was also higher in KM9 compared to *R. erythropolis*. The accumulation of lipid started at 48 h (34%) in KM9 and sustained over 72 h (36%). Where, *R. erythropolis* although represented almost similar trend but the accumulation percentage was lower as compared to KM9 that is 24% and 27% at 48 h and 72 h of incubation respectively. The growth of bot strains started to decline rapidly after 72 hours of incubation in limonene modified media. Similarly, when tested on waste as substrate the strains represented better growth as compared to limonene modified media (Fig. 7 a-b). The culture KM9 represented better growth (CDW= 610 mg L^{-1}) as compared to *R. erythropolis* (CDW= 540 mg L^{-1}). Similarly, the lipid accumulation of KM9 started at 48 h (36%) using waste as substrate and increased slightly at 72 h (38%) of incubation. Where in *R. erythropolis* the lipid accumulation varied from 26% to 32% at 48 h and 72 h of incubation correspondingly. The growth and lipid accumulation in CDW was higher in KM9 as compared to *R. erythropolis.*

3.6 Fatty acid profile

The lipids stored by bacterial cells using limonene modified media and waste as substrate were characterized through GC-Ms and are presented in Table 2. Overall, the FAME profile indicated the storage of even number fatty acids by both bacterial isolated that is KM9 and *R. erythropolis.* Where, the proportion of SFA (saturated fatty acids) is higher than MUFA (mono unsaturated fatty acids) and PUFA (poly unsaturated fatty acids). Palmitic acid ($C_{16:0}$) appeared to be most recurring compound 28% of total lipids in *R. erythropolis* (waste as substrate) followed by 23% (limonene modified media). Where in case of KM9 the proportion of $C_{16:0}$ was not greatly affected. Interestingly the production of MUFA increased using waste as substrate. $C_{14:1}$ and $C_{16:1}$ both was present in KM9 and $C_{14:1}$ was present in *R. erythropolis* only when waste was used as substrate. Where in case of KM9 $C_{14:1}$ was also present in limonene modified media ~3.15%.

4 Discussion

The strains KM9 and *R. erythropolis* were tested for their potential to grow on orange waste as substrate and their relative tolerance to limonene to evaluate their potential to grow on other citrus waste. The results indicated various physiochemical and nutritional factors that can promote cell biomass growth and thus corresponding lipid accumulation. Optimization study exhibited dependency of both strains on temperature and pH and also on carbon and nitrogen source for better growth. The optimum temperature that is 30°C for growth explains the thermophile nature of both strains (Fig. 4a-d). Both strains represented their dependence on pH, where, maximum growth was attained at 6.5 pH as compared to acidic or alkaline pH. This pH dependence could be linked to various factors like better substrate availability, and tolerance of bacterial cells to neutral pH profile [26, 27]. Where elsewhere the positive effect of increase in pH on biomass growth of *R. opacus* DSM 1069 and PD630 was observed [17]. The contradictory results from present the study could be related to isolation of KM9 [21] (Qadeer et al., 2018) on neutral pH. Similar to the physiochemical conditions both strains represent considerably affected by supplementation of carbon and nitrogen source. A promotion in growth of isolates was observed by addition of glucose and sucrose as carbon sources (Fig. 5a-b). The supplementation of media with additional carbon source could increase the assimilatory carbon. Interestingly, both glucose and sucrose are simple sugars yet the growth of both strains was more profound by the addition of sucrose where comparatively lower growth was observed by the addition of glucose and complex sugar cellulose. This could be attributed to the characteristic of isolates to use more energy efficient compound like sucrose (disaccharide) as compared to lesser efficient source. The growth increased significantly as compared to the non-augmented media. Among different nitrogen source tested NH_4Cl appeared to be most appealing source for nitrogen to promote cell growth promoting growth of both KM9 and *R. erythropolis*. Increased nitrogen availability in the media due to presence of NH_4Cl explains the limiting nutrient availability for growth (Fig. 5c-d). The results were slightly different from the previous optimization work carried out by Kumar et al. [28], where lipid accumulation and growth of *R. opacus* was more positively affected by the presence of NH_4SO_4. However, similar to this study in present work complex nitrogen source like urea represented lower growth as compared to other nitrogen source due to low hydrolysis rate [28].

The waste degradation potential of KM9 also represented its competence in removing organic matter considerably from the waste. The total degradation achieved by KM9 was greater than 50% in terms of VS and COD removal from the waste. The VS removal indicates degradation of organic matter in the waste [29, 30]. Figure 3 showed that initial removal rate was higher up to 72 h (VS removal = 27% and COD removal = 40% for OW) than gradually slow down till 96 h of incubation and further the rate declined with the progression in incubation probably due to the exhaustion of nutrients.

The removal of COD from the waste is also indication of decrease in oxidize able organic matter from the waste that is mainly due to the inoculation. This indicates that oleaginous KM9 strain can effectively utilize the waste for biomass production. This could also be linked to the lipid accumulation in KM9, as higher lipid accumulation was achieved in 72 h of incubation and declined with further progression in the experiment (Fig. 3). Previously, *Rhodococcus opacus* PD630 was used for the simultaneous dairy waste

treatment and lipid production, where the COD removal was 65% in the shake flask experiment [28], compared to 62.8% reduction observed in the present study by isolate KM9 in orange waste

R. erythropolis is reported in many studies as oleaginous therefore in present work the lipid accumulation of *R. erythropolis* is compared with culture KM9. The results indicated that KM9 accumulated more lipid as compared to *R. erythropolis.* The maximum lipid accumulation was achieved by KM9 at 72 h of incubation that is 38% using waste as substrate. Zhang et al. [30] found that *B. subtilis* H1310 can trigger lipid accumulation after 48 h of incubation; where in our previous study maximum lipid accumulation by *Bacillus cereus* was achieved at 72 h. Further in present study though lipid accumulation started at 48 hours but the property sustained and increased slightly at 72 hours indicting a prolonged stationary growth phase. This could be helpful in achieving better organic matter degradation by inoculation of KM9. At exponential growth phase the bacterial cells utilize maximum energy for growth and proliferation whereas during stationary growth phase organisms divert their maximum energy flux for critical survival of cell functions to tolerate the stressed environment [31]. Similarly, *R. erythropolis* exhibited better lipid accumulation and growth rate in waste as compared to limonene modified media. This could be related to the additional source of nutrients by supplementation of carbon and nitrogen source (NH_4Cl and Sucrose). In case of waste lipid accumulation was higher than limonene modified media because of provision of different nutrients that could play vital role in enhancing growth rate. Where the addition of auxiliary carbon in the waste ensured the lipid accumulation for longer duration and therefore the lipid accumulation sustained for longer duration. The accumulation of lipids was further confirmed by fluorescence images of KM9 in which lipids are visible at both 48 and 72 h of incubation. Where in case of *R. erythropolis* the accumulation of lipids is more visible at 72 h of incubation.

The fatty acid profile of the bacterial lipids analyzed through GC-MS (Table 2) which represented that both strains that is KM9 and *R. erythropolis* stored even carbon number lipids. This could be attributed to the neutral pH of the media as it is previously reported that bacterial isolated tend to more even chained numbered lipids [32]. In another study it was found that *R. opacus* PD630 and *Gordonia sp.* stored more even chained lipids when cultivated on waste, similar is applicable to present study. The results of present study clearly revealed that the palmitic acid ($C_{16:0}$) is present in highest proportion in the total fatty acids, (22% in KM9 and 28% in *R. erythropolis*), these results are in agreement to previous findings of Kumar et al. [28]. Interestingly, the FAMEs composition was found dependent on the type of substrate. The culture KM9 stored higher proportion of SFA using limonene modified media but the concentration in MUFA rose considerably when the same strain was cultivated using waste as growth medium. Similarly, in *R. erythropolis* no MUFA were found using limonene as media however the using waste as substrate 6% MUFA ($C_{16:1}$) was stored in cell bodies. *R. erythropolis* DCL14 is known to accumulate more PUFA [33, 34], where, in present study the degree of unsaturation by *R. erythropolis* was limited only to MUFA. This could be related to the substrate as the MUFA was only detected in waste and was absent in Limonene modified media. The presence of more SFA indicated high cloud point (CP) and pour point (PP) of the bio-oil derived from these two isolates and could have low flow properties at low temperature. The presence

of high proportion of steric acid and palmitic acid made these bacterial lipids a competent source for bio-oil production comparable to the plant based bio-oil production [35].

Previous work has been extensively done on *R. opacus* for its potential to utilize waste in addition to other oleaginous microorganisms as promising feed-stock for bio-oil production [35, 36]. However, for a sustainable, large-scale cultivation of bacteria for lipid based bio-oil production inexpensive raw material is an appealing and most desirable factor [37].

5 Conclusion

The results exhibited the potential of both KM9 and *R. erythropolis* to produce lipids, using orange waste as substrate. The competence of these strains to grow and produce lipids using Limonene represents their potential to use other forms of citrus waste also. Therefore, such type of work could be applicable to large variety of citrus family. Supplementation of the waste with minimal amount of carbon and nitrogen source has increase the CDW and also corresponding lipid accumulation. In the accumulated lipids high proportion of FAMEs showed its competence for biofuels production. The issues of low CP and PP, however, can be resolved using biofuel blends. Production of MUFA by the bacterial cells using waste showed that production of lipids and their relative composition can be modified for maximum fatty acid production and variability in chain of FAMEs according to cultivation conditions. Present work showed the potential of bacterial isolated not only to produce lipids of substantial importance but also a tool to convert waste to efficient energy products.

6 Statements And Declarations

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Competing Interests

The authors declare that they have no financial interest while execution of this study.

Author Contributions

Samia Qadeer carried out all of the experiments and wrote the first version of the manuscript. Shang-Tian Yang supervised and provided working facilities in his Chemical and Biomolecular Engineering laboratory at Ohio State University, USA. Shahid Mahmood provides technical support, particularly in the area of

bacterial isolation. Muzammil Anjum revised and copyedited the manuscript. As Samia Qadeer's PhD supervisor, Azeem Khalid contributed his expertise in the design of this work.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Novelty Statement

This study offered information regarding bacterial lipid accumulation in the new isolate for bio-oil production, as well as the added benefit of waste minimization.

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Tables

Table 1. Different waste formulation based on varying percentage proportion for growth and lipid accumulation study of KM9.

Waste Formulation	% OW	% MW	% AW
F_1	100	00	00
F_2	00	100	00
F_3	00	00	100
F_4	00	50	50
F_5	50	00	50
F_6	00	25	75
F_7	25	00	75
F_8	00	75	25
F_9	75	00	25

Table 2. Comparison of the FAMEs profile of bacterial lipids using limonene and orange waste as substrate.

Fatty acid composition (% wt/wt)		KM9		*R. erythropolis*	
		limonene modified media	waste	limonene modified media	waste
SFA	C14:0 (Myristic acid)	0.44 %	0.61%	1.25%	1.75%
	C16:0 (Palmitic acid)	22.8%	22.16%	23.25%	28.12%
	C 18:0 (Stearic acid)	15.15%	15.19%	14.547%	15.10%
MUFA	C14:1 (Pentadecanoic acid)	3.15%	1.89%	-	6.86%
	C16:1 (Palmitoleic acid)	-	2.06%	-	-
PUFA	-	-	-	-	-

Figures

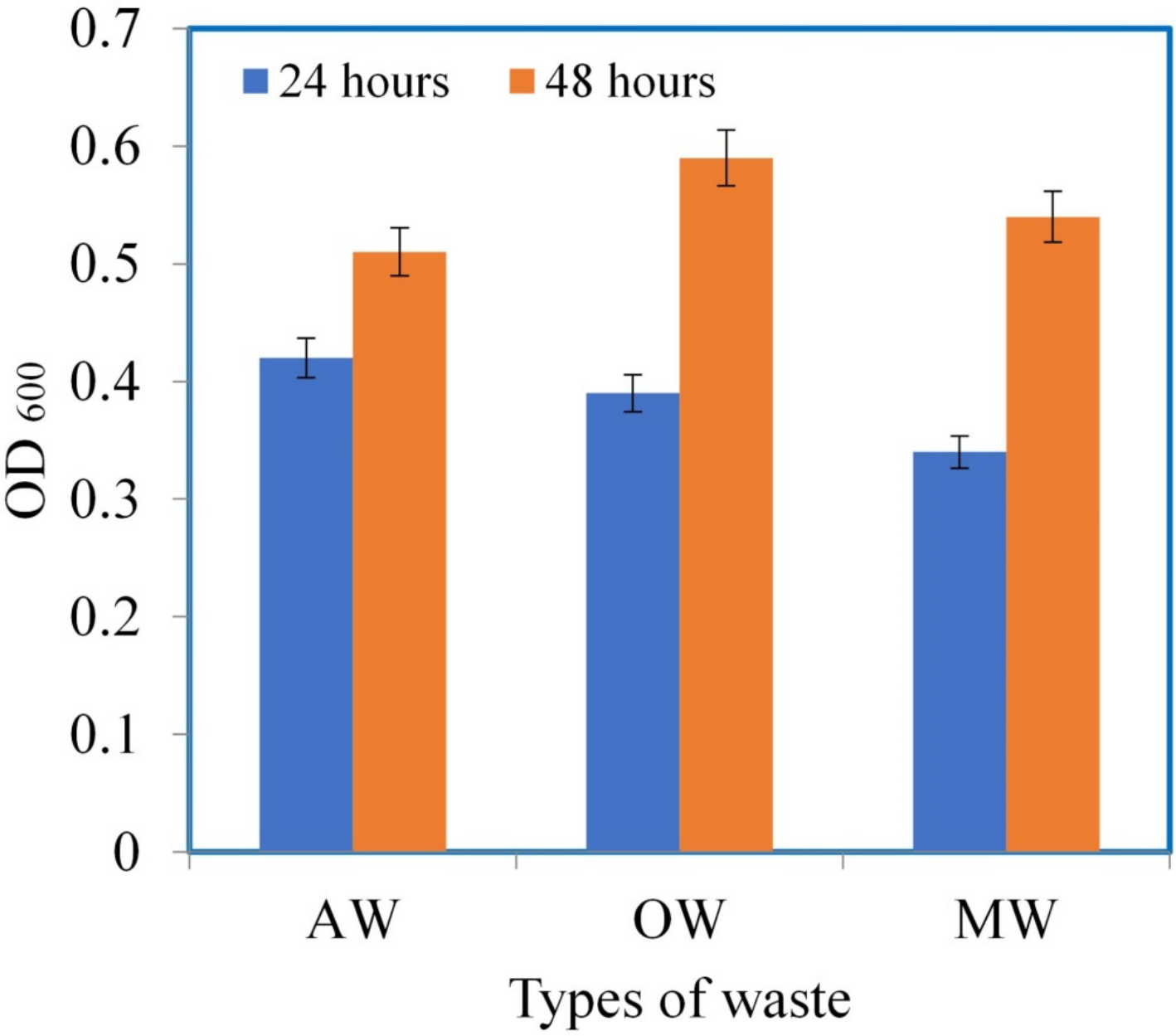

Figure 1

Potential of KM9 to use different types of waste as substrate for growth (n=3; ± S.D< 0.05).

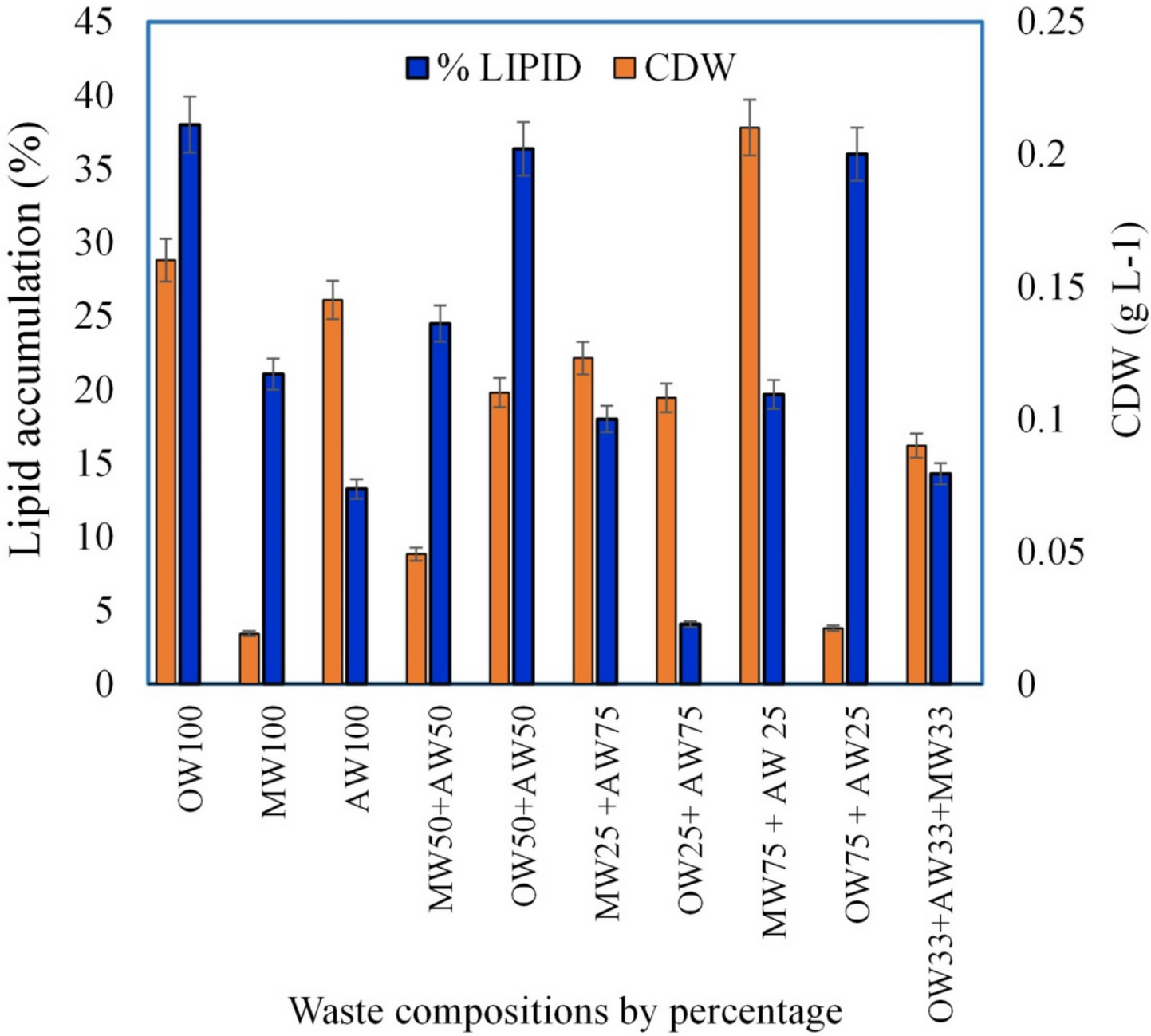

Figure 2

Potential of KM9 to use different waste types and compositions for growth and corresponding lipid accumulation.

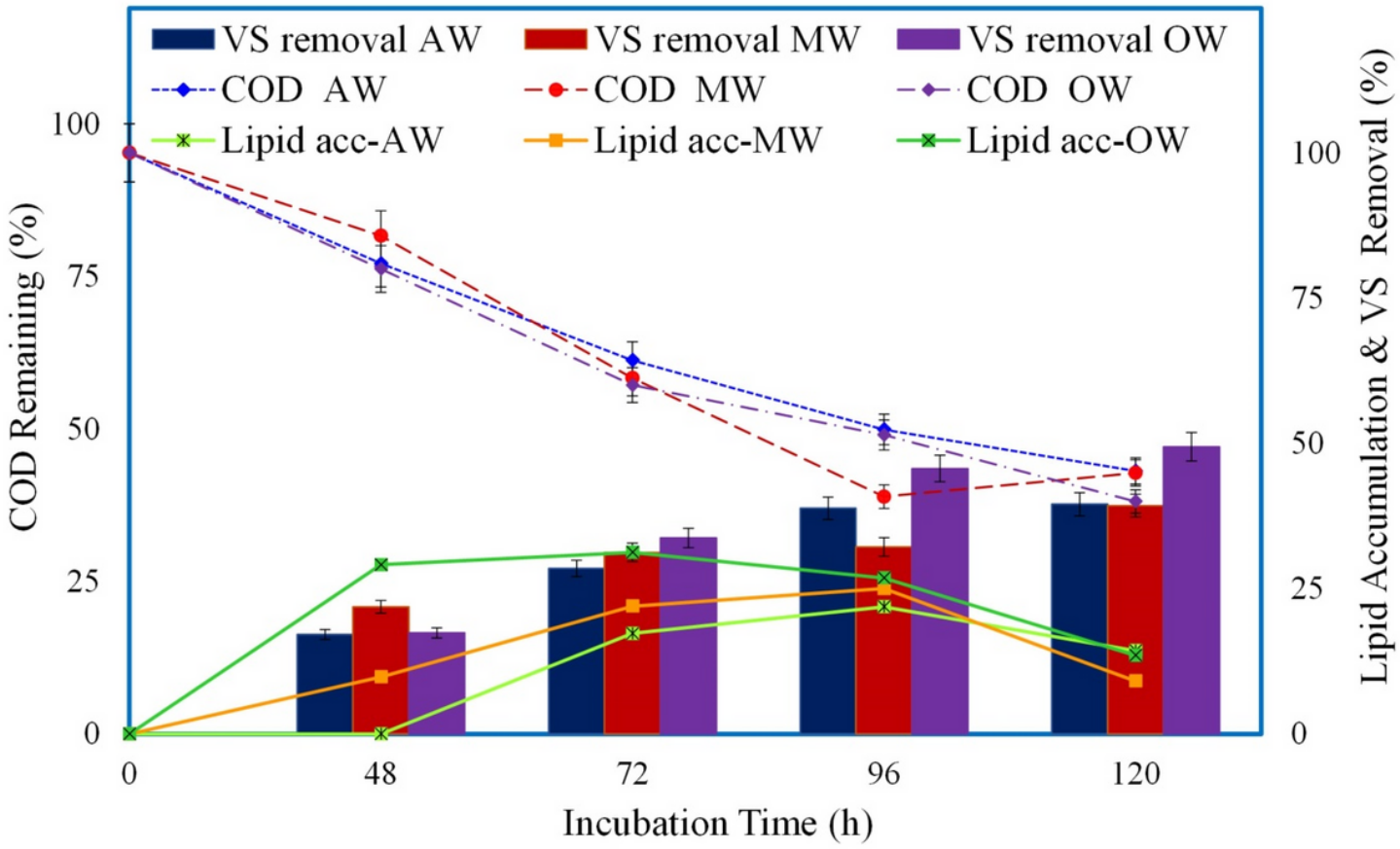

Figure 3

Organic matter (in terms of VS and COD) removal efficiency of KM9 and its potential of lipid storage using orange waste as substrate.

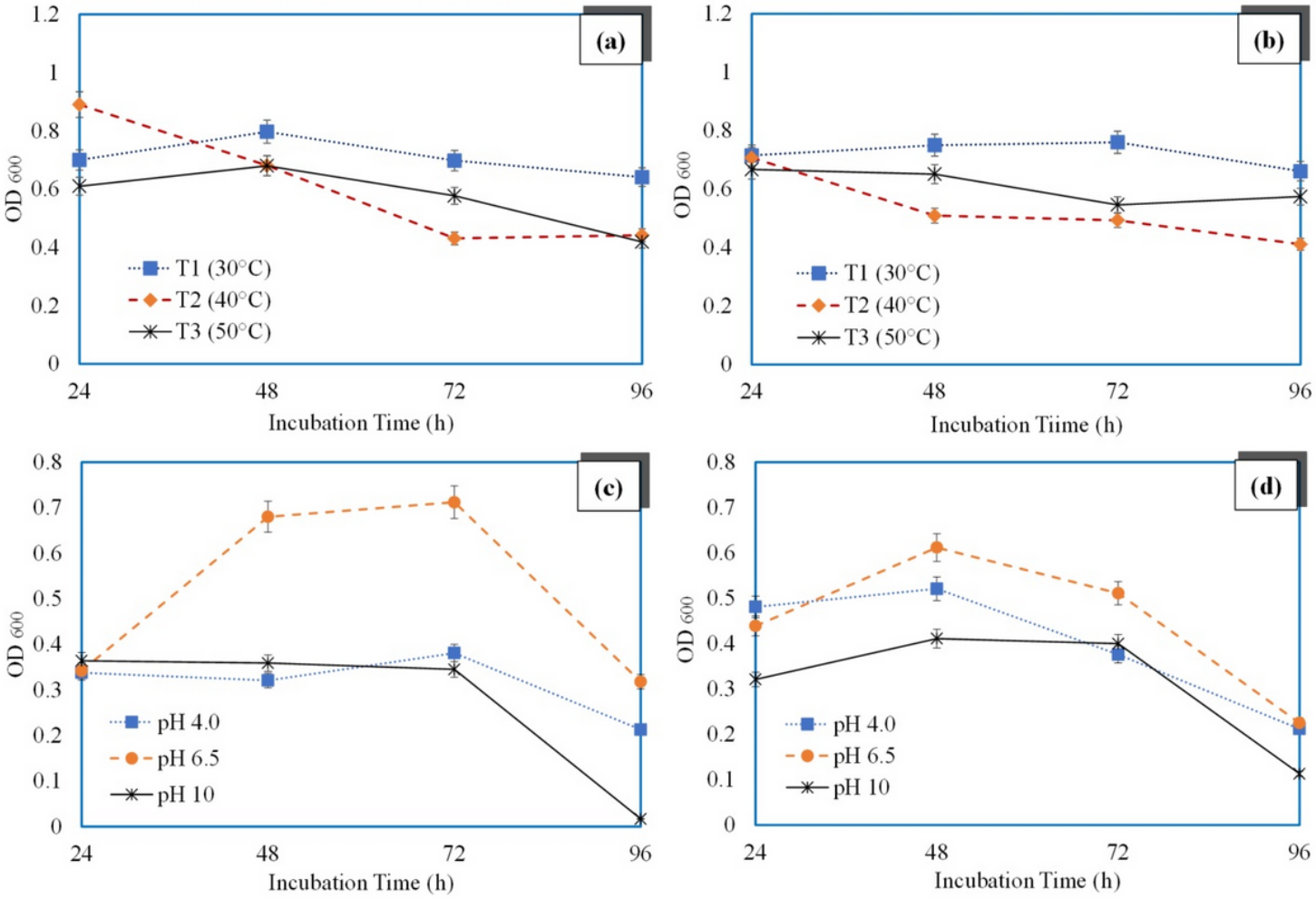

Figure 4

Optimization of physiochemical conditions for biomass cultivation of KM9 (a: Temperature; c: pH) and *R. erythropolis* (b: Temperature; d: pH).

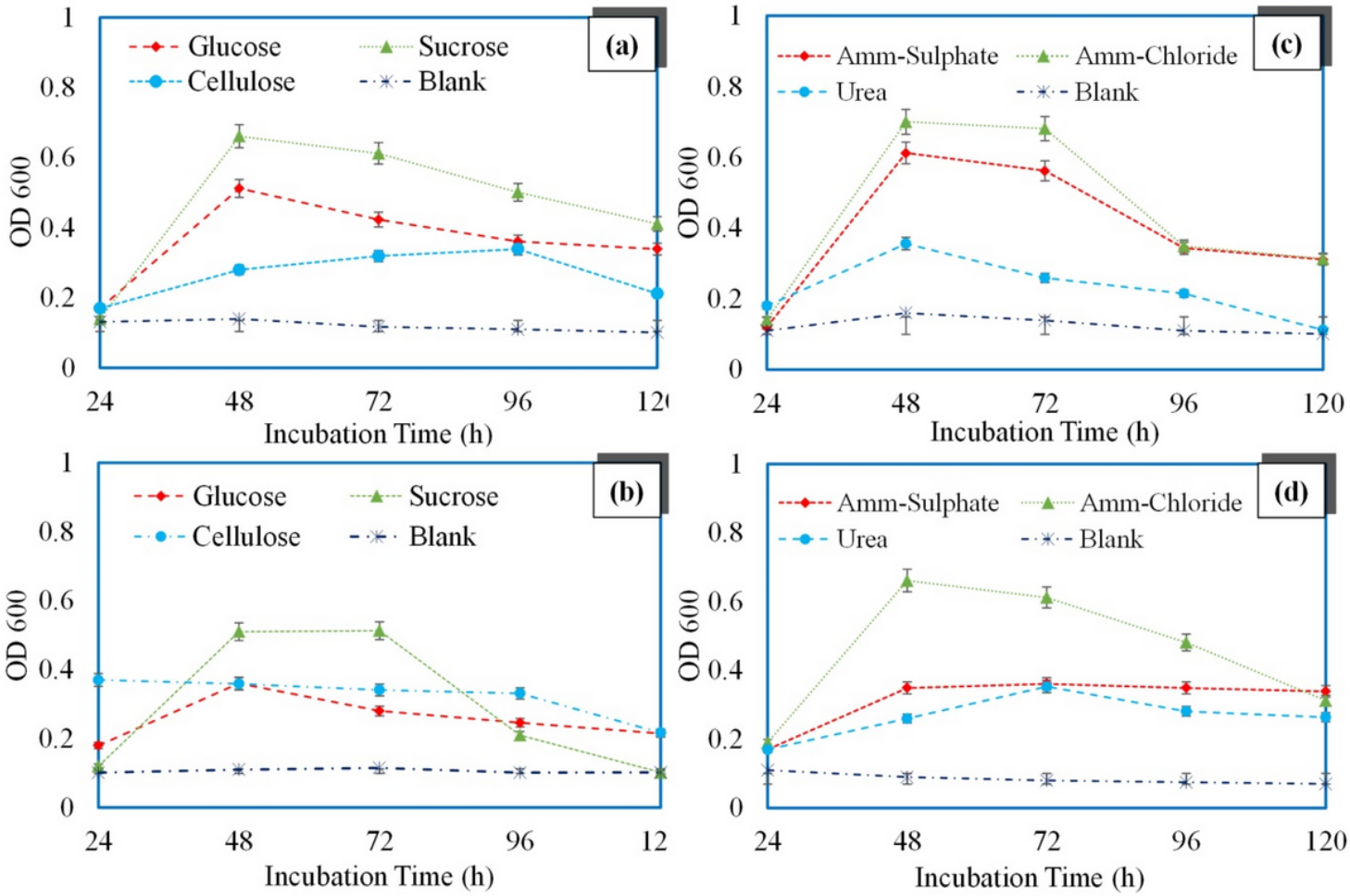

Figure 5

Optimization of carbon and nitrogen sources for biomass cultivation of KM9 (a: carbon source optimization; c: nitrogen source optimization) and *R. erythropolis* (b: carbon source optimization; d: nitrogen source optimization).

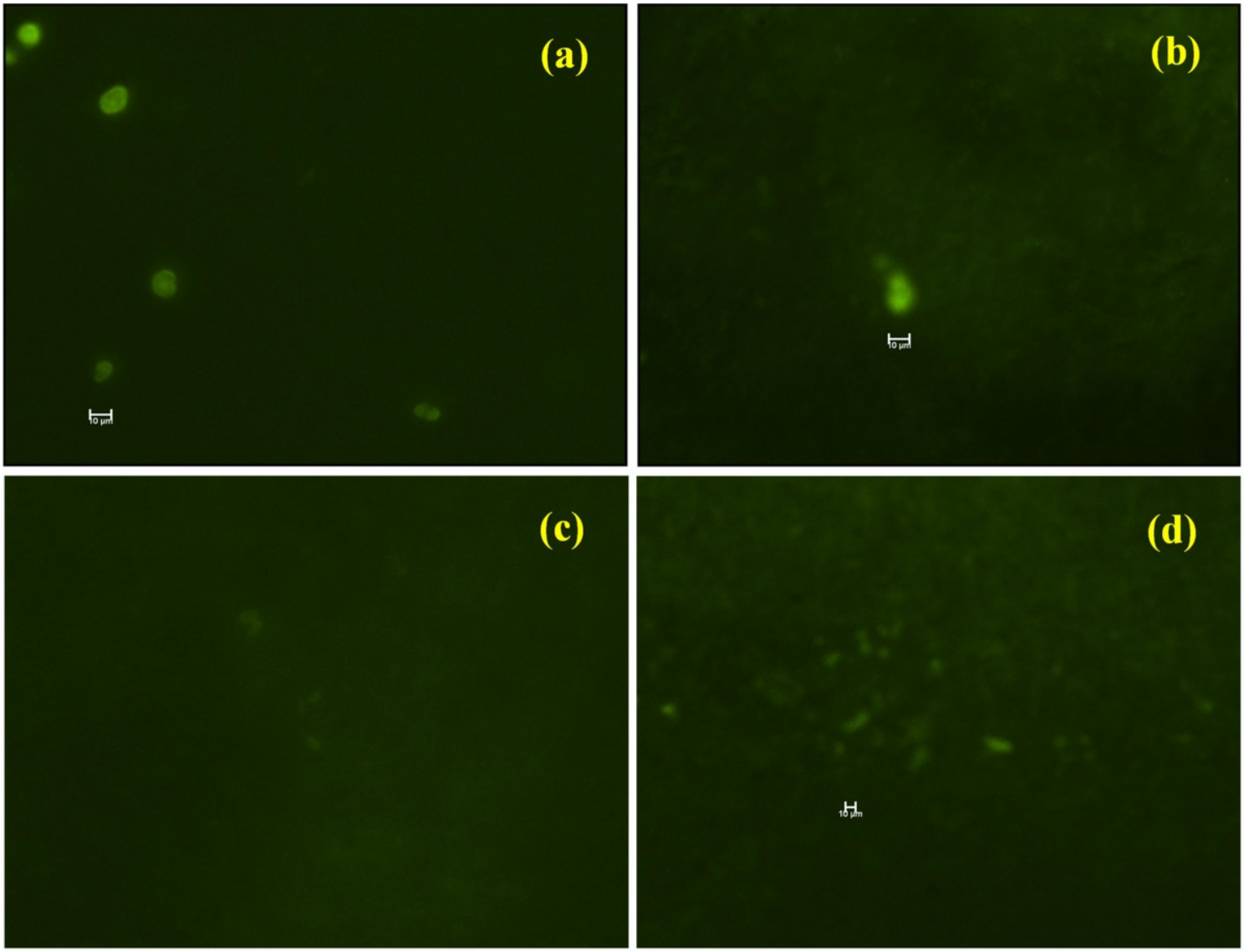

Figure 6

Fluorescence microscopy images of Nile red stained cells of KM9 (a: 48 h & b: 72 h of incubation) and *R. erythropolis* (c: 48 h & d: 72 h of incubation) sampled at stationary phase of growth.

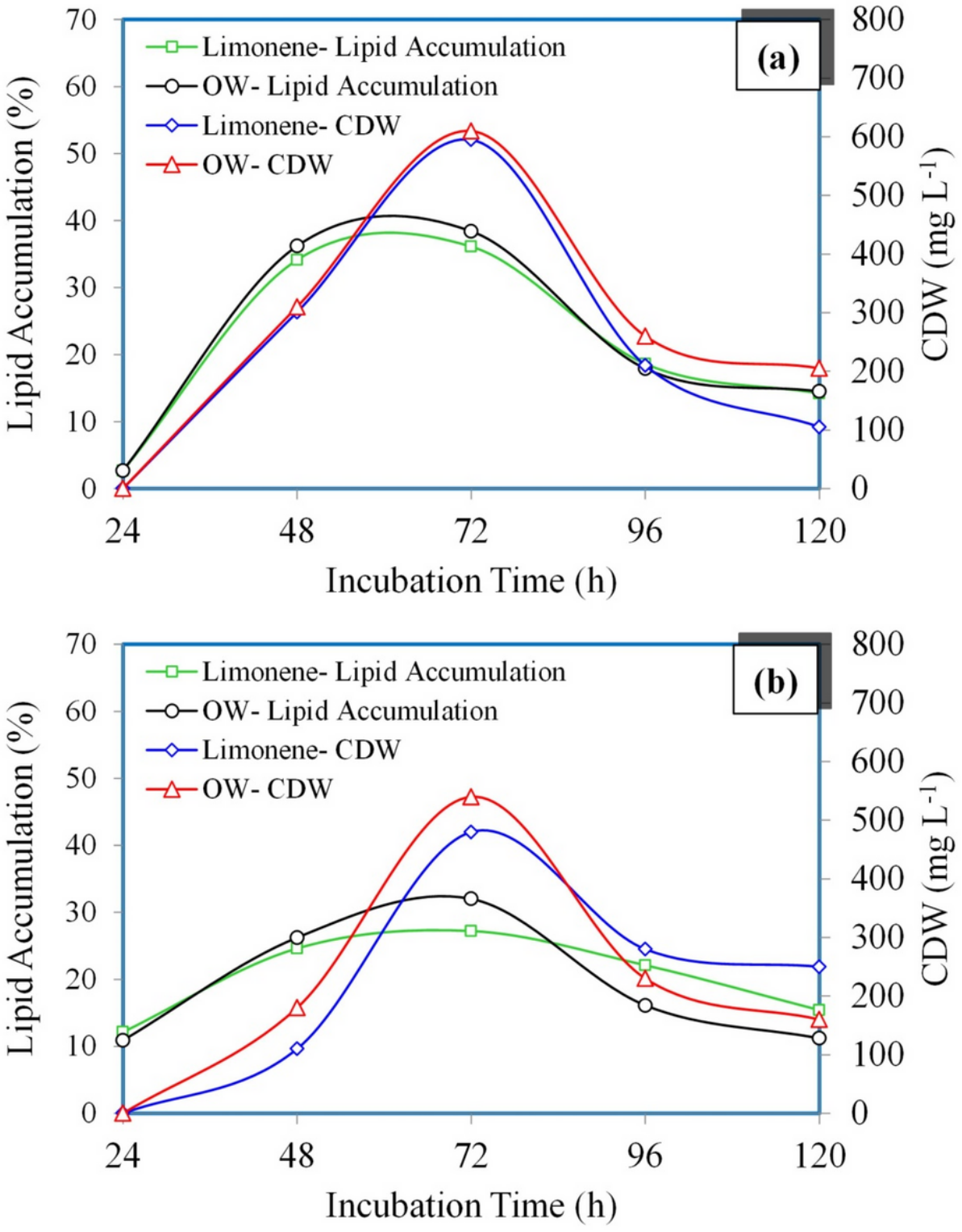

Figure 7

Time course of lipid accumulation and corresponding biomass production (a: KM9 & b: *R. erythropolis* for potential waste utilization as substrate (±S.D <0.05).

Supplementary Files

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- GraphicalAbstract.png