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Evaluation of the Lipid Composition in Activated Sludge Biomass for Biodiesel and Oleochemical Production by Thin Layer Chromatography (TLC)

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Abstract: The need to ensure that biodiesel have comparative advantage over the conventional diesel is probably responsible for the quest to reduce the cost of feedstock by investigating lipids from waste biological source like activated sludge. Usually, lipids are analysed using instruments such as gas chromatography (GC) and high performance liquid chromatography (HPLC). Unfortunately, these instruments are very expensive and are scarcely available in the developing countries. Thus, the current investigation was geared towards developing a method for the use of thin layer chromatography in determining the lipid composition in activated sludge. Upon optimization of the operating parameters, the results show that chloroform/methanol were good lipid carrier solvents, lipids were most visible using a destructive method with 5 % sulphuric acid in ethanol at 180 ^{o}C for 10 min. Petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v) and chloroform/methanol/28 % ammonia (65/25/5, v/v/v) impacted appreciably on the separation of neutral and phospholipids, respectively and plates activation was necessary for effective lipid separation. The neutral lipid classes identified were sterol, wax ester, acylglycerol, fatty acid, and the phospholipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and lysophosphatidylcholine. With this method, the lipid in activated sludge can easily be profiled, indicating a great potential for biodiesel, renewable diesel and oleochemical production.

Keywords: *Activated Sludge; Lipid; Thin Layer Chromatography; Extraction; Oleochemicals*

I. INTRODUCTION

Due to high cost of feedstock used for the production of biodiesel, concerted efforts are being made in search of cheap and available feedstock [1,2,3]. Activated sludge is one of those feedstocks, as it is a waste from the biological section of wastewater treatment plant, readily available and suspected to have high lipid contents [1,3].The majority of works carried out on the determination of lipid composition in activated sludge was conducted using gas chromatography which is very expensive. Those carried out using TLC were done without optimising the operating conditions to produce high quality results [4,5,6,7]. The current work explores the application of TLC for the analysis of the lipid fraction of activated sludge. TLC is one of the oldest chromatography and has endured due to improved reproducibility obtained using commercially available pre-coated TLC plates, and versatility of the technique resulting from a wide variety of adsorbent materials like high-performing silica[8]. Additionally, TLC possesses easy applicability, high sensitivity and speed, flexibility and cost effectiveness.It involves sample application, development and detection.It is the simplest method and has gained traction in the qualitative and quantitative analysis of complex lipids, monitoring of process reaction and purification of substance [9,10]. Despite these numerous advantages, TLC is time consuming, especially whenlarge volume of sample is required to be applied on the plate, large volume of organic solvents is needed and less accuracy of quantitative resultsmay be obtained[9,10]. These drawbacks can however be tackled by the use of high performance thin layer chromatography (HPTLC), based on the provision of sample application instrument, automatic developing chamber, TLC scanner, TLC visualizer, chromatographic immersion device, TLC plate heater and software [11]. These instruments apart from producing high quality and reproducible chromatograms are very expensive leading to increased operation cost [1]. An alternative to TLC is paper chromatography although, the latter is faster, more effective and appropriate for quantitative analysis and provides varieties of options for selection ofstationary and mobile phases [10,1]. TLC can be complemented by high performance liquid chromatography (HPLC), although, it is operated manually, has simple features and can be applied easily in the qualitative analysis of complex lipids [1]. Gas chromatography (GC) is usually being considered for volatile substances or their derivatives, with characteristics features which include extremely sensitivity, reliability and high processing speed [12]. But, like HPTLC and HPLC it is known to be expensive.

However, in this work, TLC was used to determine the lipid composition in the activated sludge biomass, due to its simplicity and cost effectiveness compared to HPTLC, HPLC and GC. The parameters which are known to influence the performance of TLC and quality of chromatograms were optimized. Such parameters include carrier solvent for lipids dissolution, effect of plate activation, method of detection and solvent systems. The optimum conditions obtained were used to analyse for the neutral and phospholipids composition in the activated sludge.

II. MATERIALS AND METHODS

A. Materials

Activated sludge biomass slurry was collected from Finham, sewage treatment works, UK. The organic solvents were chloroform (reagent plus, 99.8), methanol (99 + %), hexane (99 pure, mixture of isomers), ethanol (analytical reagent grade) purchased from Fisher Scientific UK. Others included petroleum ether (60 – 80 ^{o}C) bought from Fisons Scientific Equipment while acetone (purum, 99.0) and diethyl ether (for residual grade) were purchased from Sigma-Aldrich. Sulphuric acid (98%, analytical grade) was bought from BDH Limited Poole, England, acetic acid (purum) and ammonia solution (35%, analytical reagent grade), from Sigma-Aldrich. The neutral lipids reference standards: palmitic acid was bought from Cayman Chemical Company, oleic acid (99%), cholesterol (99 + %, GC), palmityl palmitate (≥ 99%), cetyl

palmitate from Sigma-Aldrich, respectively, mono-, di- and triglyceride mix from SUPELCO. The phospholipid reference standards: 1-monooleoyl – RAC – glycerol (C18:1, - CIS – 9), diolein (C18:1, (CIS) – 9) (dioleoylglycerol), glyceryl trioleate, L – α – phosphatidylcholine from egg yolk, type XV1-E (>= 99%, TLC, lyophilized powder), 1, 2 – diacyl-sn-glycero-3-phospho-L-serine (>= 97% TLC), L- α - phosphatidylinositol from glycine max (soybean), phospholipid mixture (for HPLC from soybean) were purchased from Sigma-Aldrich, respectively. Primuline was bought from Sigma-Aldrich.Thin layer chromatography plates (Partisil ®K6F, silica Gel 60 Å with fluorescent indication of size 20 x 20 cmwith a layer thickness of 250µm)was bought from Whatman International Ltd, England. Compact UV lamp (254nm uv/4 ~ watt, Science Company) was used to visualise the lipid spots for possible identification. TLC manual developing chamber (CAMAG) was used to provide the enabling conditions for the separation of lipid components. Memmert Oven (AtmosSAFE) was used for providing the required temperature for charring the derivatized TLC plate.

B. Sample preparation

The activated sludge slurry was dried in the Memmertoven to recover total solid biomass and the moisture content was obtained to be 1 %.

C. Lipid extraction

The lipid was extracted using Folch et al. method from activated sludge biomass [1,13]. A weight of 1 g of ground activated sludge was mixed with 20 mL of chloroform/methanol (2/1, v/v) and vortexed at 200 RPM (40.3 g) at room temperature in orbital shaker. The resulting mixture was filtered using filtration apparatus with 11 µm size filter paper. The filtrate containing extract-rich layer was washed with 4 mL of 0.9 % w/v sodium chloride in water solution to obtain the organic (containing lipids) and aqueous layers. The aqueous layer was further washed with 2 mL of methanol/0.9 % w/v sodium chloride (1/1, v/v) to recover the escaped organic layer.The volume of the organic layer was reduced at 50 ^{o}C under vacuum using rotary evaporator and then to a constant weight by a stream of nitrogen at 50 ^{o}C under atmospheric pressure. The extracted lipid was stored below 0 ^{o}C prior to analysis.

D. TLC method

The TLC plates were prewashed prior to use with chloroform/methanol (50/50, v/v) to remove any impurities capable of causing obscurity in visualising the lipids[9]. The plates were allowed to dry before activation in the Memmert oven at 120 ^{o}C for 2 .5 h. After cooling to the ambient temperature, lipids(lipid extract and lipid reference standards) were applied using Hamilton syringe followed by elution and detection. The carrier solvents for lipids, solvent system, charring temperature, charring time, derivatization reagents, plate activation and method of detection were optimised to obtain good quality chromatograms (Section 2.3.1).

1. Optimisation of factors affecting the quality of chromatograms using TLC

1.1 Carrier solvent for lipids dissolution

A concentration of 40 mg/mL of lipid extract was prepared using chloroform/methanol (2/1, v/v). Similarly, 30 mg/mL of fatty acids palmitic acid and cholesterol (reference standards) were prepared using hexane, diethyl ether, methanol or chloroform/methanol (2/1, v/v).The amount of the lipid applied on the TLC plate was 0.15 mg from each lipid solution and analysed as described in Section 2.3.

1.2 Determining the effect of plate activation on the quality of the TLC result

This was conducted using two TLC plates, with one activated in the Memmert oven at 120^{o}C for 2 h and the other unactivated. The lipid was analysed as described in Section 2.3, and 0.4 mg of lipid extract and 0.15 mg of lipid reference standards (cholesterol and triglycerol) were appliedon the plate. This was followed by elution using hexane/diethyl ether/glacial acetic acid (70/30/3, v/v/v). The separated lipid bands were visualised by spraying a solution of 5 % sulphuric acid in ethanol and charring at 140^{o}C for 40 min.

1.3 determining a suitable detecting method for lipid identification

The destructive and non-destructive methods were considered, as shown below.

(a). Destructive method of lipid detection

(i). Determining the optimum charring conditions

This was done by applying at least 0.15 mg of lipid extract and reference standards (palmitic acid, cholesterol, glyceryl trioleate and palmityl palmitate on four TLC plates. The lipids in Plates 1 and 2 were separated using hexane/diethyl ether/acetic acid (70/30/1, v/v/v) and those in plates 3 and 4 were eluted using petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v). This was followed by derivatization by spraying them with 5% concentrated sulphuric acid in ethanol and charring at varied temperatures 140 – 180^{o}C and times 5 – 120 min for lipid visualization (see Section 2.3).

(ii). Identifying a suitable derivatizing reagent

The method described in Section 2.3 was used and at least 28.72 µg of lipids (lipid extract and phospholipid standard mix) was applied on the two separate TLC plates marked A and B in triplicate.Both were developed using chloroform/methanol/28% ammonia (65/25/5, v/v/v). The lipid on plate A was derivatized by spraying it with 5% sulphuric acid (98%) in ethanol and that of plate B with 5% sulphuric acid (98%) in methanol. The plates were charred in the oven at 180^{o}C for 10 min in order to detect the lipid separated.

(b). Non-destructive method of lipid detection

The TLC plate which was earlier developed in a solvent system was sprayed with 5 mg of primuline in 100 mL acetone/water (80/20, v/v) and visualized under a UV lamp with a wavelength of 254 nm.

1.4 effects of eluent solvent/solvent system (mobile phase) on lipid separation

The impact of the elution solvent/solvent systemon the neutral lipid and phospholipid separation was determined using the method described in Section 2.3. The solvents used for neutral lipid and phospholipids are presented in Sections 2.3.2 and 2.3.3 respectively.

2. *Analysing the neutral lipid in the activated sludge*

This was done according the procedure presented in Section 2.3. Solutions of the lipid extract and lipid reference standards were prepared using chloroform/methanol (2/1, v/v) and was applied on the baseline of the four TLC plates, at the points marked A, B, C, D, E, F, G and H. The separation was done as shown in Table 1, and the lipids were visualised by spraying

with 5% sulphuric acid (98%) in ethanol and charring at 180°C for 10min except palmitic acid that was baked at 180°C for 60 min.Each lipid was eluted at least eight times and the best plates selected and combined together for presentation.

3. Identifying the phospholipids in the activated sludge

Several solvent systems such as chloroform/methanol/acetic acid/water (25/15/4/2, v/v/v/v), chloroform/methanol/acetic acid/water (50/25/7/3, v/v/v/v), chloroform/acetone/methanol/acetic acid/water (6/8/2/2/1, v/v/v/v), chloroform/methanol/28% ammonia (65/25/5, v/v/v) were investigated to obtain a solvent system that can resolve the phospholipids in the lipid extract completely for identification. The phospholipid reference standards used were L- α –phosphatidylethanolamine (PE), L- α – phosphatidylcholine (PC), L - α – phosphatidylinositol (Pl) and L - α lysophosphatidylcholine (LPC) [14,15,16]. At least 0.44 µg of the lipids were applied on the TLC and the method described in Section 2.3 was used for the separation. The lipids were visualized by spraying with 5% sulphuric acid (98%) in ethanol and charring at 180°C for 10 min.

Table 1: Operating Conditions For The TLC Analysis Of Lipids Extract From The Activated Sludge

Plates	Letters on the baseline	Lipid standards/ lipid extract	Solvent system	Amount loaded on the plate (µg)
1	A	Lipid extract Mono acylglycerols,	Petroleum ether	400
	B	Mono-, di- (1, 2-diacylglycerol and 1, 3-diacylglycerol) and triacylglycerol mix	/diethyl ether/acetic acid (80/20/1, v/v/v)	200
	C	Cholesterol		152
	D	Palmityl palmitate		238
	E	Lipid extract		400
2	F	Mono-, di- (1, 2-diacylglycerol and 1,3-diacylglycerol) and triacylglycerol mix	Hexane/diethyl ether/acetic acid (70/30/1, v/v/v)	200
	G	Lipid extract		400
3	H	Palmitic acid	Hexane/diethyl ether/ acetic acid (70/30/3, v/v/v)	150

III. RESULTS AND DISCUSSIONS

A. Optimisation of factors affecting the quality of chromatograms using TLC

The impact of the carrier solvent for lipids dissolution, solvent system, charring temperature, charring time, derivatization reagents, activation of the TLC plate and detection methods on the performance of TLC on the lipid separation was assessed to determine the optimum condition favouring the production of good quality chromatograms. The results obtained are discussed below.

1. Carrier solvent for lipids dissolution

In order to ensure that the measured lipids are transferred completely to the TLC, there is need to use a solvent which has the capacity to dissolve the lipid totally.To achieve this, the lipids were dissolved in hexane, diethyl ether, methanol and chloroform/methanol (2/1, v/v) and the method described in Section 2.3 was used for the separationwith hexane/diethyl ether/glacial acetic acid (70/30/3, v/v/v) as the solvent system. As shown in Fig. 1 all the solvents dissolved both the lipid extract and reference standardsappreciably,but, chloroform/methanol (2/1, v/v) gave a higher resolution of lipid spots after separation. This is probably due to the ability of the chloroform/methanol to dissolved total lipids (neutral and polar lipids)[17].

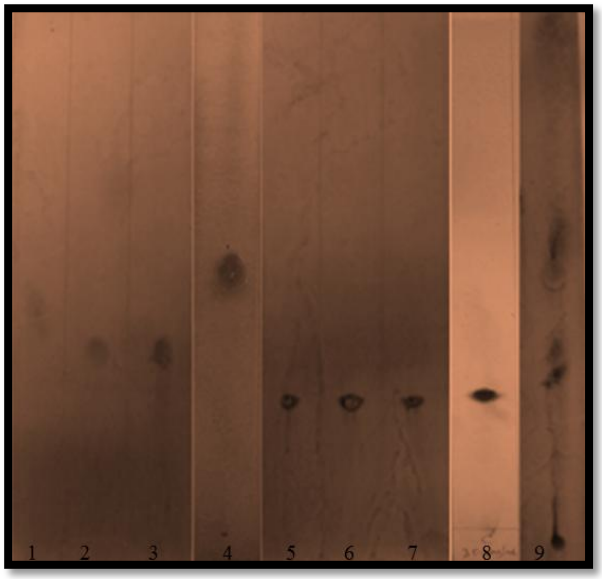

Fig. 1: effect of solvents on the resolution of the lipids chromatogram obtained from TLC (where lanes 1, 2, 3, 4 = palmitic acid dissolved in hexane, diethyl ether, methanol and chloroform/methanol (2/1, v/v), lanes 5, 6, 7 and 8 = cholesterol dissolved in hexane, diethyl ether, methanol and chloroform/methanol (2/1, v/v) and lane 9 = lipid extract dissolved in chloroform/methanol (2/1, v/v)).

2. Determining the effect of plate activation on the separation of lipid extract from activated sludge

This was carried out to investigate the significance of TLC plate activation on the separation of lipid fraction of activated sludge (lane 3, Fig. 2). As shown in Fig. 2, plate A was not activated and the lipid extract only resolved sterol (cholestrol), unlike Plate B which was activated and the lipid extract was

separated into cholesterol, triacylglycerol and other unidentified lipids. This is probably as a result of possible adsorbed moisture on plate A which could constitute a resistance to the capillary movement of lipids in the elution solvent during the chromatographic process [18]. This underscores the significance of plate activation as one of the parameters to be considered in order to achieve an appreciable lipid separation using hexane/diethyl ether/glacial acetic acid (70/30/3, v/v/v).

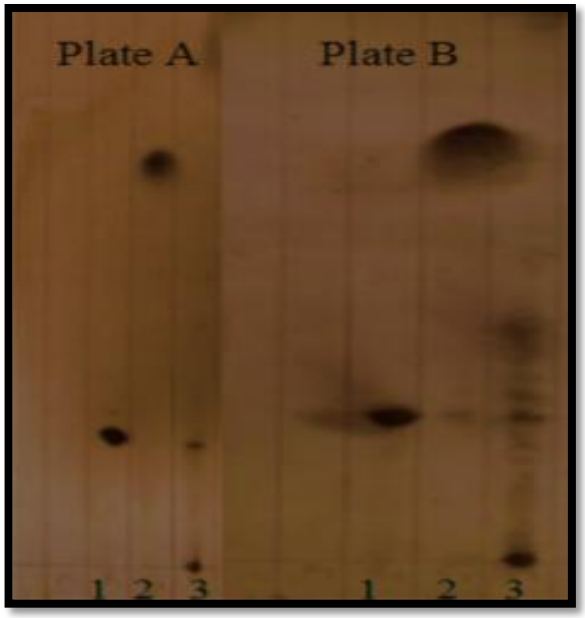

Fig. 2: effect of plate activation on the separation of lipid extract from activated sludge(where lane 1= cholesterol lipid standard, lane 2 = triacylglycerol standard lipid and lane 3 = lipid extract) (A = non activated and B = activated TLC plates).

3. Effect of detecting method on the visibility of lipid spots post TLC separation

This was conducted to evaluate the impact of destructive and non-destructive detecting methods on the lipid visibility post separation using TLC(Section 2.3.1.3). For the destructive method, four TLC plates were used and charring conducted at 140, 160 and 180^{o}C between 5 and 10 min, using 5% concentrated sulphuric acid (98 %) in ethanol, as derivatizing reagent. The results show that palmitic acid compared to other lipid standards was not visible at the conditions considered (Fig. 3 (a)). Based on this, further analysis was carried out while increasing the charring time to 70, 110, 120 min. The results show that a faint appearance of palmitic acid was observed at 140 ^{o}C and 160 ^{o}C and more obvious spot was obtained at 180 ^{o}C under the times considered (Fig. 3 (b)). Further investigation was carried out to evaluatethe potential use of 5 % concentrated sulphuric acid (98 %) in methanol to obtain a more visible lipid spots using phospholipids as the lipid standards. The plates were charred at 180 ^{o}C for 10 min and the results obtained are presented in Fig. 3 (c). As expected, visible lipid spots were observed from Plate A resulting from the use of 5 % concentrated sulphuric acid (98 %) in ethanol while faint bands of lipids were obtained using 5 % concentrated sulphuric acid (98 %) in methanol. This shows that the former derivatizing reagent is more efficient for detecting lipid separated from lipid extract using TLC compared to the later reagent.

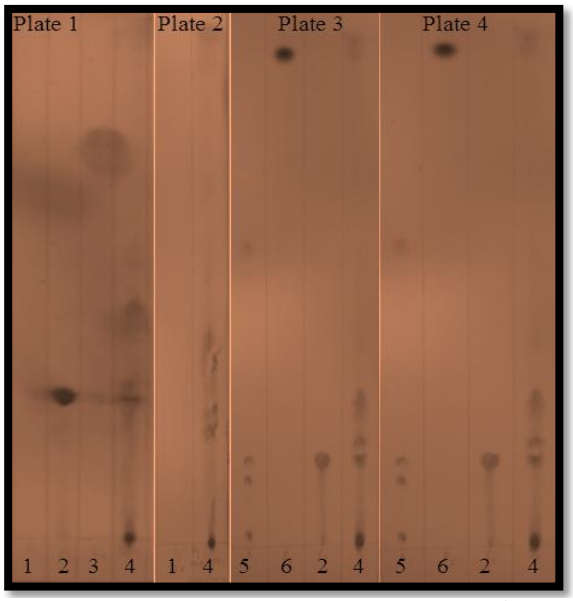

Fig. 3 (a): determining the optimum temperature and time for visualising lipids using destructive method. Where plates 1, 2, 3, 4 were charred at 140^{o}C for 10 min, 160^{o}C for 10 min, 180^{o}C for 10 min and 180^{o}C for 5 min, respectively (lane 1 = palmitic acid, lane 2 = cholesterol, lane 3 = glyceryl trioleate, lane 4 = lipid extract, lane 5 = mono-, di- and triacylglycerol and lane 6 = palmityl palmitate).

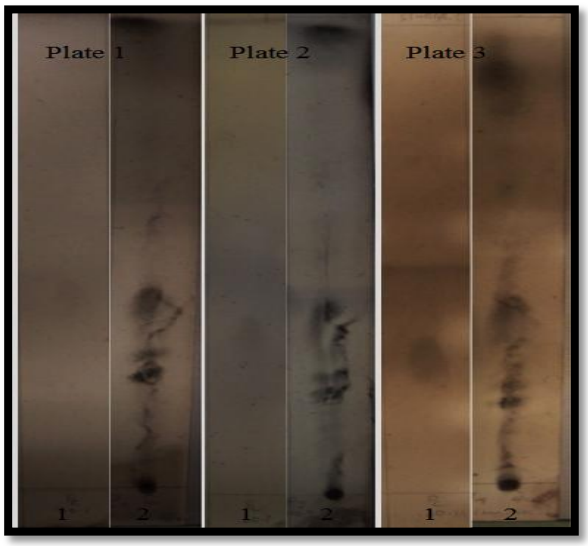

Fig.3 (b): determining the optimum temperature and time for visualising lipids using destructive method at increased charring time. Where plates 1, 2, 3 were charred at 140^{o}C for 120 min, 160^{o}C for 110 min and 180^{o}C for 70 min, respectively (lane 1 = palmitic acid and lane 2 = lipid extract).

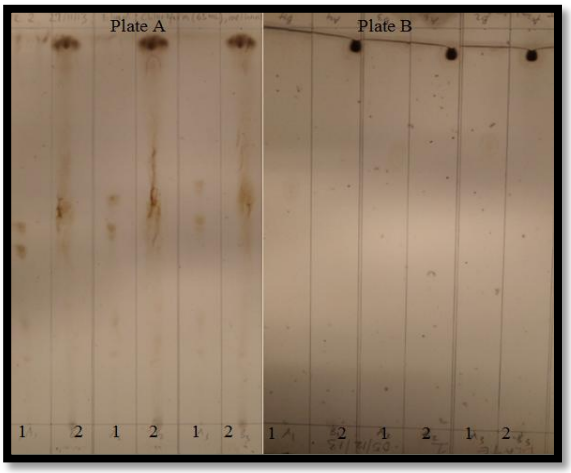

Fig.3 (c) : determining the best derivatising reagent for lipid detection at 180 ^{o}C for 10 min using lipid extract and phospholipid standard mix. Plate A - charred with 5% sulphuric acid (98%) in ethanol and plate B - charred with 5% sulphuric acid (98%) in methanol (lane 1 = phospholipid standard mix and lane 2 = lipid extract). The analysis was done in triplicate.

Conversely, no lipid chromatogram was detected using a UV lamp with a wavelength of 254 nm (non-destructive method) following the procedure described in Section 2.3.1.3 (b). This might be due to low capacity of the UV lamp used.

Hence, lipids can be best visualised by TLC under the conditions considered using the destructive method with 5% sulphuric acid in ethanol as the derivatizing reagent at 180^{o}C for 10 min.

4. effects of eluent solvent/solvent system (mobile phase) on lipid separation

This was significant in order to select a suitable solvent system which can resolve the lipid extract composition into component parts (neutral and polar lipids) in a single run, as the effectiveness of TLC is dependent on the polarity of the solvents and lipid. To achieve this, various solvents were mixed together in a specified ratio to increase or decrease their polarity, depending on the lipid class of interest in the lipid extract (see Section 2.3.1.4). The results show that petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v) exerted a great influence on the resolution of the neutral lipids and chloroform/methanol/28% ammonia (65/25/5, v/v/v) impacted on the phospholipids (see Sections 3.2 and 3.3). Although, according to Henderson and Tocher no single solvent system can completely resolve all the lipids classes in a complex lipids using a single elution[9, 1].

B. Determining the neutral lipid in the activated sludge

The lipid extract from activated sludge was analysed to assess its neutral lipid composition that might be available for bioenergy (biodiesel and renewable diesel) and oleochemicals production. This was conducted using the optimum conditions obtained in Section 3.1. The results obtained are presented in Fig. 4. The lipid classes separated from the lipid extract and the lipid reference standards are shown on the figure with the corresponding designated numbers. Sterol, wax ester and acylglycerol (monoacylglycerol) were obtained using petroleum ether/diethyl ether/ acetic acid (80/20/1, v/v/v) at points (7,10), (1,11) and (5,9).Acylglycerols (1,2-diacylglycerol and 1,3-diacylglycerol) were obtained using hexane/diethyl ether/acetic acid (70/30/1, v/v/v), at points (14, 18) and (13, 17), respectively. Free fatty acid (palmitic acid) was identified using hexane/diethyl ether/acetic acid (70/30/3, v/v/v) at points (21, 23). These results are in agreement with those presented byRevellame et al., Quemeneur and Martyand Edeh et al. using high performance thin layer chromatography (HPTLC) detergents, plastics and renewable energy [3]. The sterols are used in steroids synthesis, as surfactant, drug and renewable energy production [3]. Wax esters are used in cosmetics, lubricants, polish, waterproof and surface coating production [7]. Free fatty acids are used for soap, detergent and renewable energy production [3].

C. Ddetermining the phospholipid in the activated sludge

Like in Section 3.2 attempts were made to resolve the phospholipid composition of the lipid extract appreciably by using the following solvent systems: chloroform/methanol/acetic acid/water (25/15/4/2, v/v/v/v), chloroform/methanol/acetic acid/water (50/25/7/3, v/v/v/v), chloroform/acetone/methanol/acetic acid/water (6/8/2/2/1, v/v/v/v/v) and chloroform/methanol/28% ammonia (65/25/5, v/v/v). But, it was only chloroform/methanol/28% ammonia (65/25/5, v/v/v) that was able to resolve the lipid extract into PE, PC, PI and LPC resulting to identification using their retention factors against those of the reference standards (see Table 2). Thisresult is in agreement with that presented by Revellame et al. [4]. Edeh et al. using high performance thin layer chromatography obtained the same phospholipids composition except that LPC was not identified [2]. The reason for this could be due to the source of activated sludge biomass used and the season in which it was obtained.The applications of phospholipids are in the area ofoleochemicals and biofuels production [4]. [4,19,2]. The applications of acylglycerols include production of soap

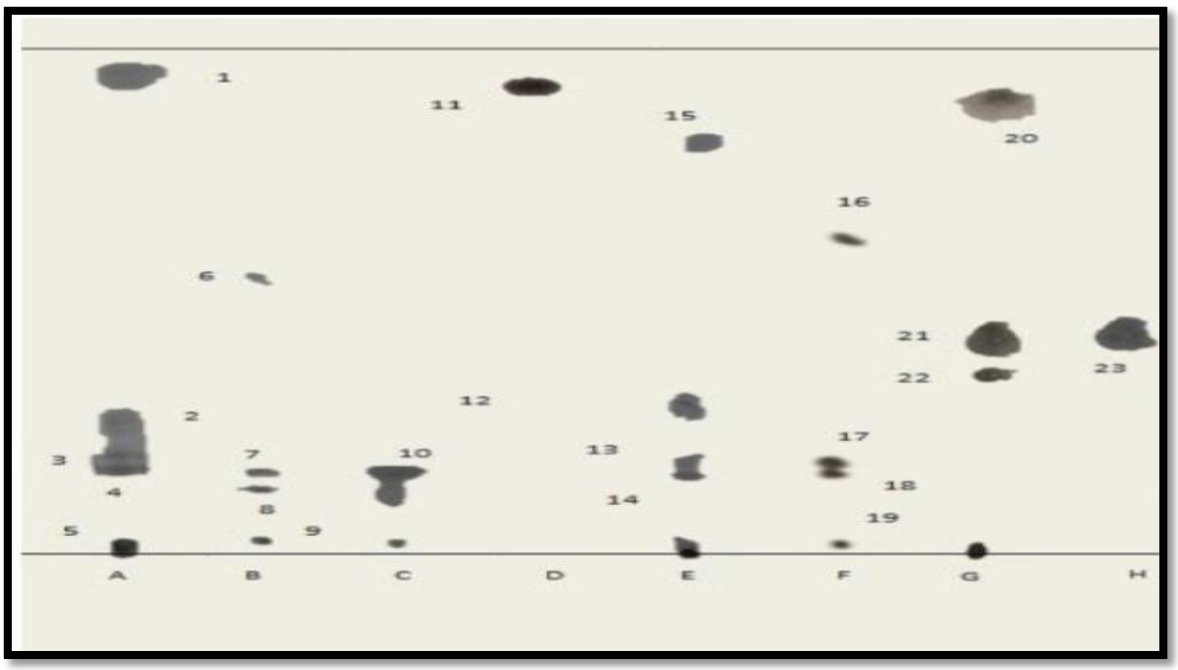

Fig. 4: determining the neutral lipid composition in activated sludge using thin layer chromatography (lanes A, E and G = lipid extract sample, lanes for lipid standards B = mono-, di- and triacylglycerol mix, C = cholesterol, D = palmityl palmitate, F = mono-, di- and triacylglycerol mix and H = palmitic acid). Cholesterol, palmityl palmitate and monoacyl glycerol were analysed using petroleum ether/diethyl ether/acetic acid (80/20/1, v/v/v, see lanes A-E), 1, 2-diacyl glycerol and 1, 3-diacyl glycerol were developed using hexane/diethyl ether/acetic acid (70/30/1, v/v/v, see lane F) and palmitic acid was eluted using hexane/diethyl ether/acetic acid (70/30/3, v/v/v, see lanes G-H). (7, 10 = cholesterol, 1, 11= palmityl palmitate, 5, 9 = monoacylglycerol, 14, 18 = 1, 2-diacylglycerol, 13, 17 = 1, 3-diacylglycerol, 21, 23 = palmitic acid). Spots 2, 3, 4, 12, 15, 20 and 22 are suspected lipids while 6 and 16 are triacylglycerol standards

Table 2: identifying the phospholipids in activated sludge using retention factor (Rf) obtained from TLC

	Retention factors (Rf) of lipids								
Solvent system	**Lipid standard (phospholipids mix)**				**Lipids extract**				
	PE	**PC**	**Pl**	**LPC**	**PE**	**PC**	**Pl**	**LPC**	**Unknown**
Chloroform/methanol/28%	0.54 ±0.02	0.45±0.02	0.24±0.01	0.16±0.01	0.55	0.44	0.2	0.15	0.975±
ammonia (65/25/5, v/v/v)					±0.02	±0.01	±0.00	±0.04	0.01

Where phosphatidylethanolamine (PE),phosphatidylcholine (PC), phosphatidylinositol (Pl) andlysophosphatidylcholine (LPC).

The lipids identified have been quantified by Edeh et al. using high performance thin layer chromatography (HPTLC) [2]. The activated sludge biomass was collected from the same source and the analysis was conducted using the optimum conditions presented in Section 3.1. The results obtained showed that the lipid content include phospholipid plusmonoacylglycerol (19.97 %),diacylglycerol (38.98 %), fatty acid (18.73), sterol (7.86%) and wax ester (4.67 %), withacylglycerol the most predominant lipid.

CONCLUSION

It has been demonstrated that the TLC method developed can be used to obtain optimum separation and visualization of the lipids in activated sludge. The lipid profile obtained has potential applications in the oleochemical industries for the manufacturing of products such as soap, detergent, cosmetics, lubricant and polish, and in renewable energy companies for the production of bioenergy such as biodiesel and renewable diesel production.

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