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Cultivation of *Mortierella alpina* on Less Expensive Medium-Chemical Evaluation of Produced Oil

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The object of this investigation apart from the recognition of the role of fatty acids is the production of arachidonic acid and its extraction from *Mortierella alpina* as the main source from powdered wasted bread which might be regarded as a good source of nitrogen and carbon for this microorganism. *M. alpina* was cultivated on a medium containing bread waste, soyabeen oil, emulsifier and mineral salts, with an initial pH of 6.5 and incubation period and temperature of 10 days at 12 °C respectively. It is expected to produce oil and arachidonic acid in respective order of 13.23 and 6.72 (g/L) after optimization of growth conditions and ingredients present in the media. The average efficiency of the oil produced from biomass was 63.54 % where the arachidonic acid (37.6 %) was the predominant fatty acid followed by palmitic (18.6%), oleic (12.72 %) and γ- linolenic (5.9 %) in respective decreasing order. Isolation of the nonsaponifiable matter of the produced oil followed by the extraction of sterol and tocopherol fractions separately indicated the presence of cholesterol, desmosterol, condisterol, 24- methyl desmosterol, 24- methylen cholesterol, lanosterol in the sterol fraction and alpha and gamma tocopherols in equal quantities in tocopherol fraction.

Key words: Arachidonic acid, Fatty acid composition, Microbial oil, *Mortierella alpina*, Powdered waste bread.

Researchers have presented wide varieties of investigations concerned with the new sources of oils and fats due to higher demands to supply the increasing population of the world as well as needs to concentrate on the quality of the oil produced[1-2]. Fungi and yeasts might be able to produce high levels of fat and oil which in term of quality might be compared to vegetable and fish oils[3-5].

Human milk contains DHA[1] and ARA[2-6] which both play important roles in infants nerves system in addition milk might be enriched with these fatty acids in order to improve its quality and consequently this has affected the I.Q[3] of the infants as suggested by Davies and Kyle [7-8]. It is well known that ARA and DHA contribute to the growth and rebuilding of tissues[9-11], therefore, interests has been increased concerning *Mortierella alpina*, one of the most industrial oleaginous microorganism that produces oil where the arachidonic acid is the predominant fatty acid[12-13].

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However due to the high cost of produced oil by *Mortierella alpina*, researchers are trying to produce oil using different and less expensive medium. It has been indicated that *Mortierella* species might be able to use different sources of carbon, namely, maltose, lactose, glucose, soluble starch, glycerol and sodium acetate as the main energy source to produce oil knowing that glucose might be considered as the main carbon source for optimization of both biomass and oil production[14-15]. In similar research work, it was conducted that glucose and yeast extract might be considered as the best carbon and nitrogen source to produce oil. Polymers of glucose namely, starch and dextrin was also effective to act as carbon source in oil production[16]. In order to decrease the cost of the finished product, compost and sunflower seed oil was employed to cultivate *M. alpina*, although the nutrients in the compost that used by this fungi were not specified[17,18]. Park *et al.*[19], indicated that replacement of yeast extracted by soya meal as the nitrogen source has improved the yield of ARA which might be due to feather like morphology of *M. alpina* in this medium.

Result from the previous research has shown that the enrichment of the medium with flour and vegetable oil enables improved growth and consequently increase the production of *M.alpina*[20]. Soyabean oil, a popular vegetable oil with its particular resistance to pH was employed to carry out the research work[21]. Bajpai has suggested a process for the production of ARA where a medium containing glucose, soya flour and inactivated baker's yeast extract was employed. The results have been shown that after 65.5 h incubation a biomass (22 g/L) and ARA (2.3 g/L) were obtained. It was observed that the species tends to grow in the form of the pellet which is a disadvantage due to the growth rate and decreased yield of ARA[22]. The medium suitable for the growth of *M. alpina* must contain sources of carbon, nitrogen and organic salts. Source of carbon and nitrogen might increase the price of the media or finally the finished product, therefore it is the object of this investigation to replace some carbon sources (glucose) with less expensive material (powdered waste bread) containing sufficient concentration of carbon.

MATERIALS AND METHODS

Microorganism and culture condition

M. alpina CBS 343/66 was obtained from the Fungal and Yeast Collection of the Institute of Royal Netherlands Academy of Arts and Sciences and was maintained on potato dextrose agar slants at 4 °C and transferred every 3 months. Potato dextrose broth was employed as inoculums and was prepared in 500 mL baffled flasks containing 100 mL medium. The culture was grown for 3 days at 26 °C with shaking at 150 rpm. Cultures were inoculated with 10 % (V) inoculums and incubated on a shaker for 10 days at 28 °C or 12 °C and 150 rpm. All medium components were separately heat sterilized (121 °C) by autoclave. The medium contained (g/L): glucose 100, yeast extract 11, KH_2PO_4 3.8, $NaNO_3$ and $MgSO_4.7H_2O$ 0.5[23]. The mycelium was harvested by vacuum filtration, washed by distilled water and freeze dried.

Analytical methods

Biomass concentration was determined gravimetrically. Total lipids were determined by hexane extraction at 40 °C. Fatty acids methyl esters were prepared according to the method recommended by the American Oil Chemist's Society (AOSC); Ce 1-62[24]. Fatty acids were analysed by the application of the methyl esters using by GC- MASS according to the method described by Eroshin *et al.*[25]. Fatty acid composition was used to determination the iodine value or degree of unsaturation by the following formula:

Degree of unsaturation = 1(% of monoene) + 2 (% of diene) + 3 (% of triene) + 4(% of tetraene) + 5 (% of pentaene) + 6 (% of hexaene)[26] ...(1)

Lovibond apparatus was employed to determine the red, yellow and blue colors of the extracted oil according to the standard AOCS; Cc13e-92. The nonsaponifiable matter of the oil was isolated by saponification of the oil with alcoholic potassium hydroxide followed by the extraction of the nonsaponifiable matter with ether according to AOAC method; 933.08[27]. The Non saponifiable matter of the oil was fractioned into different classes of compounds on 0.5 mm thickness of silicagel G type 60 plates developed in hexane: ether (4:1) and finally sprayed with 0.01

% rhodamine 6G in ethanol and visualized under UV lamp. The sterols fraction was firmly identified, removed from the plate and extracted by ether and finally applied to GC-MASS apparatus according to Cunha *et al.*,[28]. Saponification number was calculated from fatty acid composition according to AOCS method; Cd 3-25 [2]). Phosphorus representing the concentration of phospholipid was measured according to the method presented by Hudson and Ghavami[30]. Induction period of the extracted oil, representing it's resistant to oxidation was determined by Metrohm Rancimat model 743 apparatus measuring the secondary oxidation products of oil and fats at 120 °C. High Performance Liquid Chromatography was employed to identify and quantify tocopherols according to Barnes and Taylor[31]. Sterols and fatty acid methyl esters standard, were purchased from Sigma chemical company of USA. Solvents and other chemicals used where purchased from Merck chemical company of Germany.

Statistical analysis

In this study, the Taguchi method and Qualtek4 were used to find the optimal condition for growth of *M. alpina*[32].

RESULTS AND DISCUSSION

Selection of appropriate and inexpensive source of carbon

In order to select proper source of carbon initial assessment were made to evaluate corn flour, sucrose, date extract and powdered waste bread to replace glucose and soya flour originally present in the media as described by Higashiyama *et al.*[23] and Jie Jin *et al.*[33].

Date extract as a carbon source

The media containing (g/L) 144 date extract, 11 yeast extract, 3.8 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 3.4 $NaNO_3$ where the date extract replaced glucose according to Jie Jin *et al.* [23]. As shown in Fig. 1 the mean rate of produced biomass was 0.8 (g/L) which indicated the absence of *M. alpina* growth since in the medium describe the date extract contained 70 % glucose. It was thought that the replacement of original glucose with this substance might have encouraged the growth, but this did not occur due to the presence of some inhibitory component in the extract.

Powdered waste bread as a carbon source

In this investigation similar medium was employed but powdered waste staled bread was replacing the soya flour and glucose in the medium

Table 1. Condition of treatments and average of oil biomass and ARA production

Treatment	Waste bread (%)	Ammonium Nitrate (%)	Temp. (ºC)	Soy Oil (%)	Tween 80 (%)	Biomass (g/L)	Oil (g/L)	ARA (g/L)
1	5	0	12	0	0	17.19	7.34	3.08
2	5	1	12	0.1	0	15.98	3.01	1.33
3	5	2	28	0.2	0.001	12.34	2.04	0.79
4	5	0	28	0	0.001	11.99	5.97	2.04
5	10	0	12	0	0.001	16.73	10.48	4.61
6	10	1	12	0.2	0.001	31.53	7.16	3.78
7	10	2	28	0.1	0	19.29	10.56	4.37
8	10	0	28	0	0	19.38	6.60	2.62
9	15	1	28	0	0.001	15.15	9.74	4.08
10	15	1	28	0	0.001	17.05	2.96	1.07
11	15	2	12	0	0	33.10	4.19	1.60
12	15	0	12	0.2	0	10.94	1.98	0.88
13	5	0	28	0.2	0	18.36	5.90	2.57
14	5	1	28	0	0	24.94	7.09	2.75
15	5	2	12	0	0.001	9.01	4.13	1.80
16	5	0	12	0.1	0.001	27.95	19.30	9.28

Table 2. Oxidation resistance and iodine value of some sources of oil[50-51]

Oil	Induction period (h)	Iodine value
Grape seed oil	2.2	142
Sunflower oil	2.2	127
Corn oil	3.7	115
Canola oil	3.8	118
Tallow	4.4	48
M. alpina oil	4.5	197
Sesame oil	4.6	105
Olive oil	5.2	83
Soybean oil	5.3	140

formulation[33]. The amount of biomass and oil produced as indicated in Fig. 1 was evaluated (39.85 and 17.63 g/L respectively).

Sucrose as a carbon source

M. alpina was cultivated on a medium as describe previously but glucose was replaced by sucrose. Most microorganisms prefer to use simple sugar such as glucose but few might be able to use disaccharides and polysaccharide such as sucrose and starch. *M. alpina* is able to use sucrose and produce oil although a medium containing glucose is preferred. It should be noted that this fungi is able to use starch as a carbon source but due to the gelatinization of starch at high concentration which

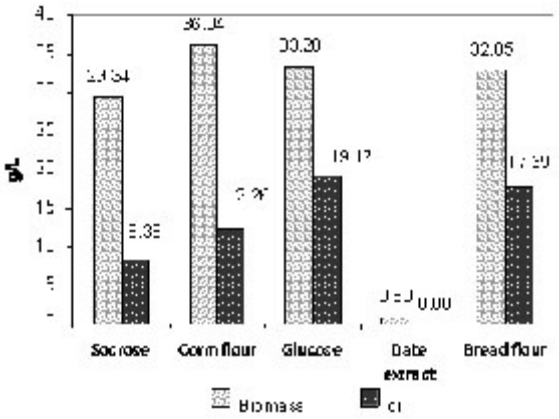

Fig. 1. Biomass and oil production of *M. alpina* in medium with different carbon sources

occurs after sterilization might create problems which inhibits the growth to a great extent with final reduction in biomass and oil produced.

Corn flour as a carbon source

Corn flour has been employed as the carbon and nitrogen sources to grow *M. alpina*. It is shown that the amount of biomass is higher than other experiments when different source of carbon were employed but the amount of produced oil covered only a third of biomass meaning lower oil production. Due to the fact that glucose and yeast extract containing medium produced the highest amount of oil, it was decided to adopt a situation similar to above medium therefore cluster analysis was used to find the right similarity and relation.

According to Fig. 2, concerning the oil production it might be concluded that the medium containing glucose fallowed by powdered waste bread, corn flour and sucrose are suitable and their suitability decreases respectively as a mentioned. Considering the economical point of view, it is worth to mention that the medium containing powdered waste bread is regarded the least expensive source of nitrogen and carbon.

Iran is ranked first in the world regarding the amount of bread wasted annually. Other countries namely turkey[34], United Kingdom and United State have similar problems in term of wasted bread[35,36]. Due to the high amount of wasted bread one might not focus on this compound as a waste or lost but to consider it as a valuable raw material to be included in the media for possible production of valuable compounds[37,38].

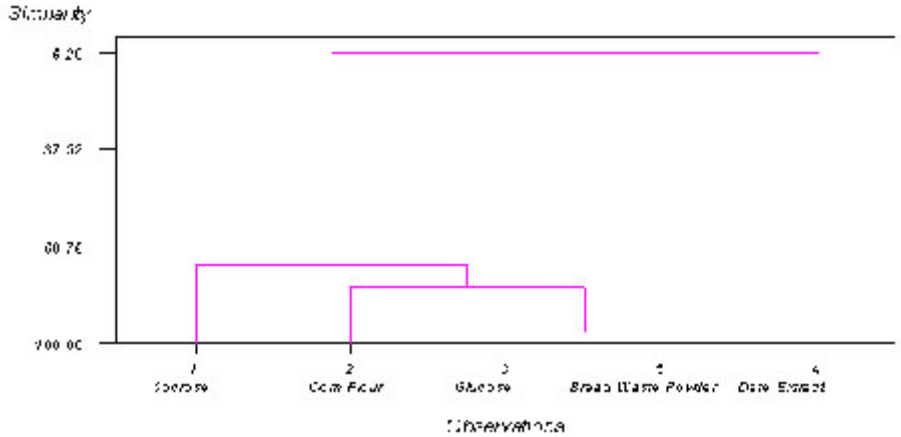

Fig. 2. The cluster analyze of biomass and oil produced by *M. alpina* in different carbon sources. The vertical axis shows the similarity percentage of mediums

Optimization of oil production in medium containing powdered waste bread

In order to find the best condition for production of oil and ARA, three levels of carbon source (5, 10, 15 % as powdered waste bread), three levels of nitrogen source (0, 1, 2 % as ammonium nitrate) at two different temperatures (12 and 28 °C) and three oil supplement (0, 0.1 and 0.2 % soyabean oil) and two levels of emulsifier (0, 0.001 % Tween 80) were employed and evaluated according to Taguchi method where sixteen treatments were carried out in duplicate order. Ten days after cultivation, the oil was evaluated and the amount of ARA was determined (Table 1).

Determination of the best condition for producing oil

Fig. 3 shows the effect of powdered waste bread, ammonium nitrate and soyabean oil on the production of oil. The amount of oil produced is increased as the level of powdered wasted bread is increased but at higher concentration of powdered waste bread this path way is not followed as there is a reduction in the oil produced which might be due to the osmotic condition provided when there is a high concentration of carbon source. Further to the natural characterization of ingredient present in the bread, namely starch which gelatinizes at high temperature casing an extreme increase in the viscosity and consequently decreases the availability of nutrients and oxygen which are vital compounds for the growth of *M. alpina*. This obstacle is more severe at low temperature (12 °C). Presence of ammonium nitrate as a source of nitrogen at different concentrations indicated that the highest amount of oil is produced when this compound was absent. A high ratio of carbon to nitrogen is required to encourage the biosynthesis of oil[39,40]. Therefore nitrogen concentration must be limited for better accumulation of the oil.

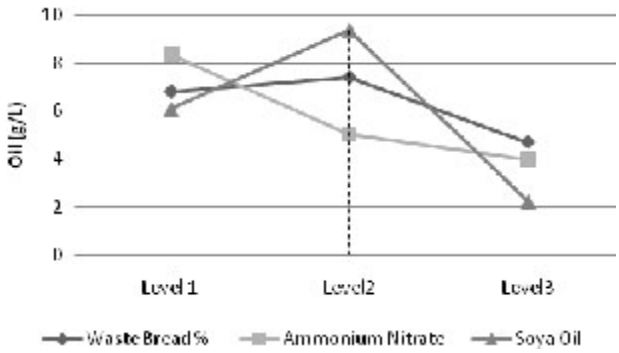

Fig. 3. Main effect of three level factors on oil production

The presence of soyabean oil at 0.1 % concentration has increased the oil production approximately 1.6 times as this substance was absent. This is due to biological pathway for oil production since the microorganism might use the fatty acids present in soyabean oil as the substrate to produce longer and more unsaturated acids[41].

Fig. 4 shows the effect of two combined factors, the temperature and Tween 80 on the oil production. The results indicated that oil production at lower temperature has increased which agrees with the research carried out previously by Zychae and Siepmnn[42], Gam[43], Domsch *et al.* [44].

The addition of soyabean oil with emulsifier improves the productivity, since the emulsifier reduces the surface tension between the water and oil phases. Variance analysis of the average oil produced indicated that ammonium nitrate and soyabean oil was the two major influencing factors affecting the oil production. Other factors; the emulsifier, powdered waste bread and temperature were less effective in respective decreasing order.

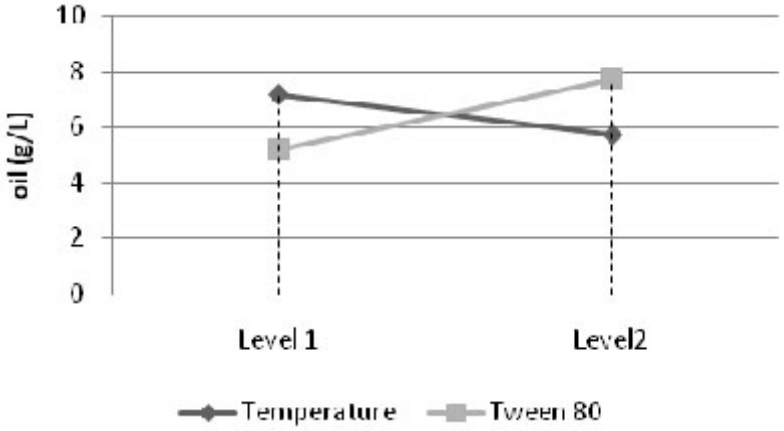

Fig. 4. Main effect of two level factors on oil production

Determination of the optimum condition for the production of ARA based on Taguchi method

It has been shown that the production of ARA depends on the amount of the oil produced and the productivity of the acids where 32-48 % of the total fatty acid present is ARA

As shown and indicated in Figs. 5 & 6, the factors investigated which were most effective in the production of oil were also effective in production of ARA. The additional of soyabeen oil at the level of 0.1 % has encouraged the production of ARA in high quantity which agrees with the work carried out by Jang *et al.*,[45], Cohen and Ratledge[46].

According to the analysis of variance and interaction studies, the factors affecting the oil production also have the same effect on the ARA production. It has been predicted and might be expected that the cultivation of *M. alpina* in a medium containing 10 % powdered waste bread, 0.1 % soyabean oil, 0.001 % emulsifier and mineral salt namely $MgCl_2$. $6H_2O$, KH_2PO_4, $CaCl_2$. $2H_2O$ and Na_2SO_4 in the absence of excess nitrogen with an initial pH of 6.5 and incubation period of ten days at 12 °C and 150 rpm might produce 14.31 (g/L) oil and 7.32 (g/L) ARA.

To confirm the predicted value and evaluate the results, tests were carried out according to the condition described earlier in triplicate order. The results indicated an actual yield of 92.45 and 91.80 % for oil and ARA which these values are at most 10 % lower than what were expected. Therefore the medium containing powdered waste bread might be considered as the least expensive source of nitrogen and carbon to produced oil and consequently ARA.

Chemical evaluation of the oil produced by Mortierella alpina

The oil produced by *M. alpina* was subjected to series of tests to evaluate its quality and the results were compared to some vegetable oils. The tests concerned consisted of the

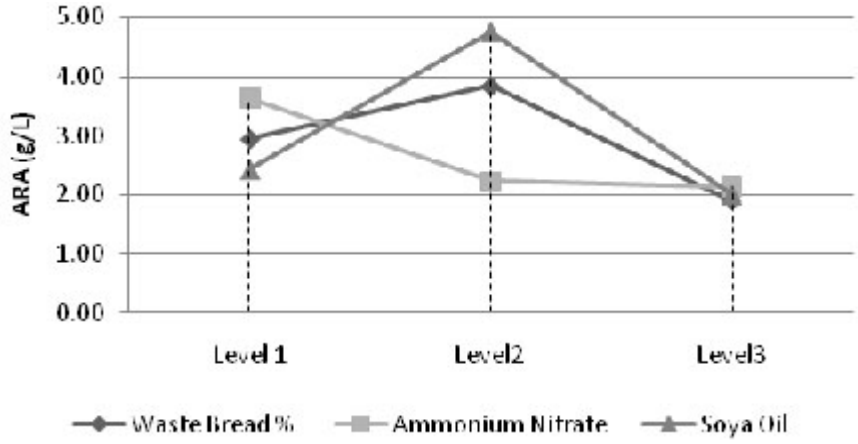

Fig. 5. Main effect of three level factors on ARA production

determination of fatty acid composition, iodine and saponification values, color measurement, nonsaponifiable matter and phospholipid contents, induction period measurement representing the resistance of oil to oxidation and qualitative and quantitative determinations of tocopherols and sterols.

The fatty acid profile of the oil produced by *M. alpina* indicated that ARA was the predominate fatty acid present. Other acids namely palmitic, oleic, stearic, myristic, γ- linolenic, behenic, lauric, homo gamma linoleic, margaric and nervonic were present in different quantities. The presence of γ- linolenic which is highly valued nutritionally might be considered quite important in the oil produced. The fatty acid EPA is produced at lower incubation temperature therefore if the oil is intended to be used in infant food formulation, the production of this fatty acid should be stopped meaning the application of higher incubation temperatures[46]. The iodine value of the produced oil at 28 °C which is the reflection of its unsaturation is 197 ($^{g\ Iodine}/_{100\ g\ oil}$), indicates that the oil produced is quite sensitive to oxidation chain reaction, even more sensitive than linseed oil which contains a considerable quantity of α- linolenic acid.

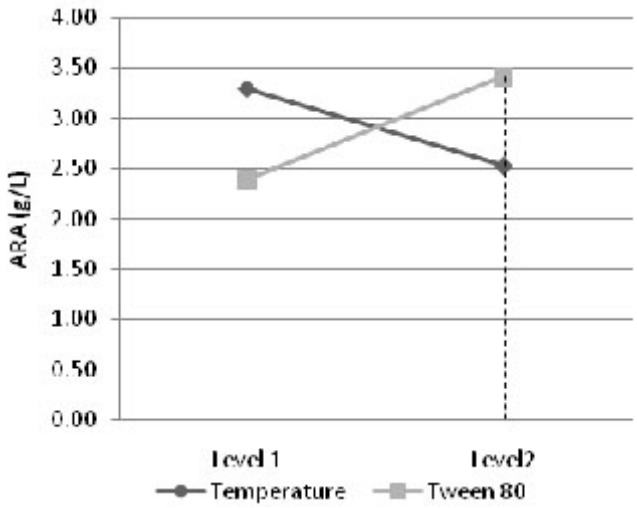

Fig. 6. Main effect of two level factors on ARA production

Amounts of Saponification Value of oils other than *M. alpina* are written from Lewkowitsch and Warrburton[47].

The saponification value of the oil representing the mean value of the fatty acids molecular weights present in the triglycerides is 186 ($^{mg\ KOH}/_{1g\ oil}$). This value is lower than those described for most of the vegetable oils due to the high content of ARA except rapeseed oil which contains considerable amount of erucic acid (Fig. 7). Examination of the oil by Lovibond apparatus indicates yellow, red and blue colors of 11.3, 3.7 and 0.05 in respective order. The intensity of yellow, blue and red colors of the produced oil is much less than crude vegetable oils but quite similar to rice bran oil. Changes of color in oils produced were experienced which might be due to the use of different media and incubation conditions. The changes might be considered as a subject of further investigation, but bleaching process might be applied to all the oil produced to provide a uniform product.

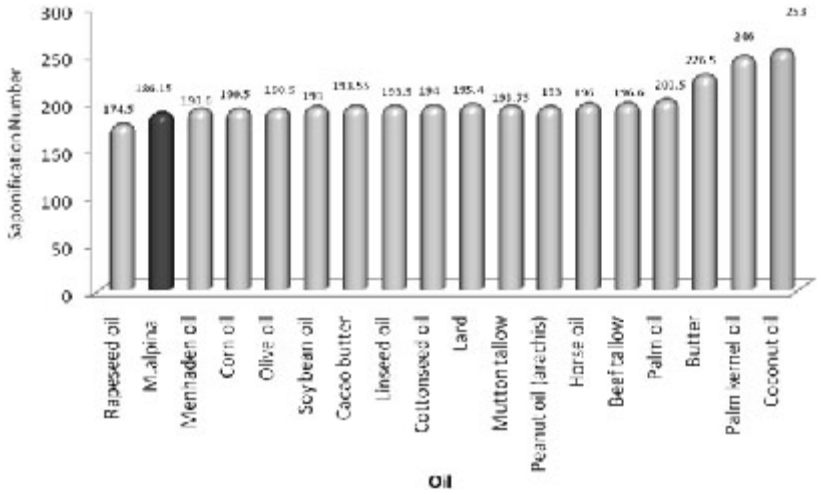

Fig. 7. Saponification value of vegetable and *M. alpina* oils

Fractionation of the nonsaponifiable matter on TLC plates indicated that sterols, tocopherols and hydrocarbons were the major fractions present. Qualitative and quantitative determinations of sterols indicated the presence of desmosterol the predominant sterol followed by lanosterol, 24- methyl desmosterol, condisterol and 24- methylene cholesterol in respecting decreasing order. Trace of cholesterol was identified. β- sitosterol, the major the predominate sterol in all vegetable oils was not present and identified in the oil produced[48,49]. The predominant sterol, desmosterol behave quit similar to cholesterol but due to the quantity present, the effect might be considered negligible. Equal quantities of tocopherols, α (60 ppm) and γ (69 ppm) were identified in the oil produced; both have vitamin E and antioxidant activities. The amount of tocopherols present in the oil might be considered the optimum concentration for antioxidant activity protecting the oil against oxidation. The bleaching operation recommended earlier for this oil might affect both sterols and tocopherol contents to some extent[50]. The induction period measurements representing the stability of the produced oil indicated an induction period of 4.5 h at 120 °C which is quite remarkable for such substrate contain considerable quantities of ARA and γ- linolenic acids in the presence of tocopherols therefore further investigation might be required to understand or identify other compounds which might be present. Further work on the oil indicated that phospholipids were present at very low concentration (108 ppm). Therefore the undesirable possible effects namely flavour and color changes due to the presence these compounds might not be experienced.

CONCLUSION

Medium containing glucose and yeast extract might be considered superior for oil production, among the alternative mediums, the most suitable and the least expensive medium is the one containing powdered waste bread. The results showed that powdered waste bread is a source of carbon and nitrogen and when extra nitrogen source was added the production of oil was reduced intensively. Enrichment cultures with soyabean oil and emulsifier might increase the production of oil and consequently the acids. The oil resulted from the growth of *M. alpina* is aimed to be used as a part of infants food formulation, therefore slight refining operation to reduced some compound namely coloring and flavouring matters might be required to uniform the final product.

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