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Biological studies on the housefly, *Musca domestica* L. treated with essential oils

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Abstract
This present study shows the potential of five different essential oils Citronella oil, Ylangylang oil, Clove oil, Lemon Grass oil and Thyme oil, showed biochemical activities against *Muscadomestica* larvae. Newly hatched 2nd instar larvae were collected from the mass culture maintained under the laboratory condition. The larval bioassay was evaluated by using dipping method Protein estimation was determined according to Modified Lowry's method. The total lipids were estimated by Knight *et al*. (1972) method. DNSA Method was used to determined amylase activity. In the present investigation the total protein, lipid and amylase contents levels are higher in 3% concentration when compared with that of 1% and 2% concentration of each essential oils. The total protein, lipid and amylase in the supernatant homogenated 2nd larval instar of *M. domestica* larvae was highly decreased as compared to control groups at all different concentrations. These changes indicate that various biochemicals specific changes occur at different concentration. The present findings seem promising and therefore suggest that the usage of essential oils are more effective to reduce the protein, lipid content and amylase activity in 3% concentration.

Keywords: *Musca domestica*, clove oil, biochemical, larvae, and bioassay

1. Introduction
The Housefly *Musca domestica* L. is a worldwide known pest/vector causing public health problems (West, 1951) [114]. Their role as vectors of human and animal pathogens especially those responsible for enteric diseases are due to the habit of defecation and regurgitation on animal and human food (Howard, 1911) [43]. It spreads disease causing organisms, especially *E. coli*, Shigella and Salmonella spp. (Ahmad *et al.,* 2007; Holt *et al.,* 2007; Nayduch and Stuzenberger, 2001) [4, 41, 70]. Houseflies disperse to areas of human habitation and activity from areas commonly found around human and animal waste (Mian *et al.,* 2002; Sulaiman *et al.,* 2000) [6, 104]. Such notorious house flies known for their ability to develop resistance mechanisms to avoid and detoxify chemical insecticides. Resistance to DDT was detected in short duration after its introduction (Varzandeh *et al.,* 1954; Perry, 1958) [108, 81]. There flies also developed resistance to organophosphates, carbamate and pyrethroid insecticides (Boxster and Campbell, 1983; Plapp, 1984; Kaufman *et al.,* 2001b; Butler *et al.,* 2007) as well as growth regulators such as diflubenzuron and cyromazine (Shen and Plapp, 1990; Bloomcamp *et al.,* 1987). Due to continuous use of pesticides resulted in efficacy losses (Sheppard *et al.,* 1990) and development of cross resistance (Scott, 1989).

1.1 Effects of essential oils
Plants are well known producers of diverse kind of chemical compounds and essential oils that are used for defend plant against different kinds of pests (Isman and Akhtar, 2007). Various properties such as killing and repelling pests, affecting insect growth and development, antifeedant and arrestant effects, antifungal, antiviral and antibacterial action against pathogens, were evaluated (Prakash and Rao, 1986, 1997). Acetyl cholinesterase inhibition and octopaminergic effects were detected after treatment of essential oils as fumigant (Isman, 2000). Also effects on behavior modification and contact toxicity for different life stages were evaluated by Koul *et al*. (2008). Palacios *et al*. (2009 a,b) studied the effects of 21 medicinal and edible plants oils against Housefly in which he detected limonene (92.5%) and 1,8-cineole (56.9%) as principle components of orange peel and eucalyptus leaves. Medicinal plants with pulegone, menthone, limonene and 1, 8-cineole were found most toxic to house fly. In another study 34 plants were evaluated for fumigant and toxicity efficiency by Pavela (2008). The principle components of peppermint oil were menthone and menthol (Palacios *et al.,* 2009 b). The plant tissues extracted, climatic and growth conditions, variation in cultivation and the methods used for extraction and analysis affects the composition of oils from particular species. For this reason there have been considerable efforts to examine the effects on individual components that are common to those essential oils known to have insecticidal properties (Isman, 2000; Koul *et al.,* 2008).

1.2 Protein
According to Walstra *et al.,* (1999) milk contains dozens of other types of proteins beside the caseins. They are more water-soluble than the caseins and do not form larger structures. Because these proteins remain suspended in the whey left behind when the caseins coagulate into curds, they are collectively known as whey proteins. Whey proteins make up approximately twenty percent of the protein in milk, by weight. Lactoglobulin and proteose-peptone are the most common whey protein by a large margin.

1.3 Amylase
Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms (Banks et al, 1975). They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolysing α-1, 4- glycosidic bonds of amylose, amylopectin, glycogen and their degradation products (Damien et al, 2010). They act by hydrolysing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved (Dhanya et al, 2009). In recent years a number of new enzymes associated with degradation of starch and related polysaccharides structures have been detected and studied (Mohammad et al, 2010).

1.4 Lipids
Lipids are one of the major constituents of foods, and are important in our diet for a number of reasons. They are a major source of energy and provide essential lipid nutrients. Nevertheless, over-consumption of certain lipid components can be detrimental to our health, *e.g.* cholesterol and saturated fats. In many foods the lipid component plays a major role in determining the overall physical characteristics, such as flavor, texture, mouth feel and appearance. For this reason, it is difficult to develop low-fat alternatives of many foods, because once the fat is removed some of the most important physical characteristics are lost. Finally, many fats are prone to lipid oxidation, which leads to the formation of off-flavors and potentially harmful products.

From all above it is evident that plant originated essential oils can prove great beneficial for pest/vector control. The present work aims at the assessment of bioactive potential of essentisal oils which found to have Anti-inflammatory, Spasmolytic, Antiparasitic, Antibacterial, Anti-arthritic, carminative, Laxative, Tonic, Diuretic properties (Warrier et al, 1994), against the Housefly. Data obtained was subjected to statistical analysis.

2. Materials and Methods
2.1 Rearing of animal *Musca domestica*
Housefly larvae were cultured on natural feed the cow dung. Humidity of the feedstuff was 70%±5% and temperature of about 25-30° C. The housefly eggs were introduced into the feed for further hatching. The containers were all closed for odor control. In the containers: the temperature was maintained at 28±2° C., and the humidity at 70±5%.The feedstuff was stirred once a day to avoid overheating and internal oxygen shortages after placing fly eggs in the feed stuff. The interior of the containers were maintained in the dark with darkness. Housefly larvae were produced within one rearing cycle of 3 to 3.5 days for each container. (Plate 1 & 2).

2.2. Larvicidal activity
Newly hatched 2nd instar larvae were collected from the mass culture maintained under the laboratory condition. Ten 2nd instar larvae were taken in a container. The container contains sample mixture. The larval bioassay was evaluated by using dipping method (Sinthusiri *et al.,* 2013). The larvae were dipped into 10 ml of each test solution for 30 sec. Then transferred them to a filter paper (in plastic box, size 7.5X10.0X7.5 cm). Larval mortality was recorded at 1.0, 6.0, 12.0 and 24.0 h. The criteria for mortalities were evaluated that larvae of house flies not responding were considered dead. Each test was performed in five replicates with positive control and negative control (ethyl alcohol).

2.3 Biochemical assays
The pre-pupal known weight larvae were exposed to test oils for 24 hours. The dead larvae were collected from the test. The treated larvae were used for homogenization. The larvae were homogenized in 20ml of phosphate buffer of pH 6.8.The samples were centrifuged at 8000 rpm in Ultra Centrifuge (Remi-CM12) for 10 minutes at 40ºC. The supernatant was used for biochemical assays. (Plate 6).

2.3.1 Estimation of Amylase activity by DNSA method
DNSA Method was used to determined amylase activity (Plummer 1988, Sumner 1925).

Dinitrosalicylic Acid Reagent (DNS Reagent)
Dissolve by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 mL 1% NaOH. Store at 4°C. Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.40% Rochelle salt solution (Potassium sodium tartrate).

Procedure
- Take 0.5 ml of enzyme extract.
- Add 0.5 ml of substrate (1% Starch) and 0.5 ml phosphate buffer (pH 6.8, 0.2 M).
- This mixture was incubated for 10 minutes.
- Add 1ml DNSA reagent.
- The mixture was then kept in boiling water bath for 10 minutes.
- After this it was cooled, diluted to 10 ml and optical density (OD) was read at 540 nm on UV spectrophotometer (Systronics-Model 119 PC Based).
- For each assay four replicates were taken.
- Data was obtained and tabulated.

2.3.2 Estimation of protein by Lowry's method
Protein estimation was determined according to Modified Lowry's method (Raghuramalu*et al.*, 1983).
A. 2% Na2CO3 in 0.1 N NaOH
B. 1% NaK Tartrate in H2O
C. 0.5% CuSO4.5 H2O in H2O
D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C
E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water
BSA Standard - 1 mg/ ml

Procedure
- Take 0.1 ml of larval extract.
- Add 0.5 ml of alkaline copper reagent in each tube.
- Mixture was incubated at room temperature for 10 min.
- Add 2 ml of phenol reagent.
- These tubes were heated for 5 minutes at 55°C and were cooled under running water.
- OD was measured at 650 nm on UV spectrophotometer

(Systronics-Model 119 PC Based).
- Standard curve was used to calculate amount of protein using bovine serum albumin as standard.
- For each assay four replicates were taken.
- Data was obtained and tabulated.

2.3.3 Estimation of the total lipids
The total lipids were estimated by the method of Knight *et al*. (1972).

Phosphovanillin reagent
Pure vanillin (0.6 g) was dissolved in 10 ml ethyl alchohol and completed to 100 ml with distilled water. 400 ml of concentrated phosphoric acid was added, and the solution was stored in dark glass bottle at room temperature.

Procedure
- 250 µl of samples solution was added to concentrated sulphuric acid (5 ml) in a test tube and heated in a boiling water bath for 10 min.
- After cooling to room temperature, the digest (500 µl) were added to phosphovanillin reagent (6.0 ml).
- After 45 min, the developed colour was measured at 525 nm against reagent blank prepared from 500 µl distilled water and 6.0 ml phosphovanillin reagent.
- The result is expressed as µg lipid / insect.
- For standard curve, serial concentrations of lipids (oleic and palmitic acids at a ratio of 7:3) from 1 to 5 µg were prepared in absolute ethanol (concentration of 1 to 5 µg/ml) were used treated in the same manner as the unknown.
- For each assay four replicates were taken.
- Data was obtained and tabulated.

4. Results
The results presented in (Tables 1-6) exhibit the biochemical aspects of essential oils Citronella oil, Ylangylang oil, Clove oil, Lemon Grass oil and Thyme oil, showed biochemical activities against *Musca domestica* larvae respectively (Plate 7)

Table 2 shows that the treatments of 2nd instar larvae of *M. domestica* with different concentrations of the Citronella oil. The total protein, lipid and the amylase activity was estimated. The lowest value observed in total protein, lipid and amylase activity more effective in 3% concentration, reached 0.435 ± 0.01 mg/g, 0.265 ± 0.0058 mg/g and 0.123 ± 0.0126, respectively compared with 0.67 ± 0.012, 0.34 ± 0.014 and 0.348 ± 0.032 for control.

Table 3 shows that the treatment of 2nd instar larvae of *M. domestica* with different concentrations of the Ylang Ylang oil. The total protein, lipid and the amylase activity reached its lowest value observed more effective in 30% concentration, reached 0.475 ± 0.006 mg/g, 0.138 ± 0.005 mg/g and 0.148 ± 0.005, respectively compared with 0.67 ± 0.012, 0.34 ± 0.014 and 0.348 ± 0.032 for control.

Table 4 shows that the treatment of 2nd instar larvae of *M. domestica* with different concentrations of the Clove oil. The total protein, lipid and the amylase activity reached its lowest value observed more effective in 30% concentration, reached 0.358 ± 0.015, 0.243 ± 0.0096 and 0.148 ± 0.005, respectively compared with 0.67 ± 0.012, 0.34 ± 0.014 and 0.348 ± 0.032 for control.

Table 5 shows that the treatment of 2nd instar larvae of *M. domestica* with different concentrations of the Lemon Grass oil. The total protein, lipid and the amylase activity reached its lowest value observed more effective in 30% concentration, reached 0.393 ± 0.010, 0.045 ± 0.006 and 0.053 ± 0.010, respectively compared with 0.67 ± 0.012, 0.34 ± 0.014 and 0.348 ± 0.032 for control.

Table 6 shows that the treatment of 2nd instar larvae of *M. domestica* with different concentrations of the Thyme oil. The total protein, lipid and the amylase activity reached its lowest value was observed more effective in 30% concentration, reached 0.423 ± 0.030, 0.025 ± 0.010 and 0.083 ± 0.005, respectively compared with 0.67 ± 0.012, 0.34 ± 0.014 and 0.348 ± 0.032 for control.

The above results reveal that various changes occur in the total protein, lipid and amylase contents in the 2nd instar larvae. These changes indicate that various biochemicals specific changes occur at different concentration. In the present investigation protein, lipid and amylase contents levels are higher in 3% against the 2nd instar larvae when compared with that of 1% and 2% concentration of each essential oil.

Table 1: Biochemical components were estimatedin housefly larvae

Control	
Biochemical components	**Optical density values**
Protein (mg/gm Body Weight)	0.67 ± 0.012
Lipid (mg/gm Body Weight)	0.34 ± 0.014
Amylase (µmole/ml/min)	0.348 ± 0.032

Table 2: Biochemical components were estimatedin housefly larvae against citronella oil.

Biochemical components	Citronella		
	OD		
	1%	2%	3%
Protein (mg/gm Body Weight)	0.6375 ± 0.021	0.5425 ± 0.030957	0.435 ± 0.01
Lipid (mg/gm Body Weight)	0.43 ± 0.0231	0.3625 ± 0.0263	0.265 ± 0.0058
Amylase (µmole/ml/min)	0.367 ± 0.015	0.255 ± 0.0192	0.123 ± 0.0126

Table 3: Biochemical components were estimatedin housefly larvae against Ylang ylang oil.

Biochemical components	Ylang Ylang		
	OD		
	1%	2%	3%
Protein (mg/gm Body Weight)	0.623 ± 0.013	0.528 ±0.010	0.475 ± 0.006
Lipid (mg/gm Body Weight)	0.348 ± 0.015	0.245 ± 0.024	0.138 ± 0.005
Amylase (µmole/ml/min)	0.345 ± 0.010	0.265 ± 0.010	0.148 ± 0.005

Table 4: Biochemical components were estimatedin housefly larvae against clove oil.

Biochemical components	Clove OIL		
	OD		
	1%	2%	3%
Protein (mg/gm Body Weight)	0.525 ± 0.0058	0.4275 ± 0.0096	0.358 ± 0.015
Lipid (mg/gm Body Weight)	0.245 ± 0.0192	0.14 ± 0.0142	0.243 ± 0.0096
Amylase (µmole/ml/min)	0.3225 ± 0.010	0.015 ± 0.006	0.148 ± 0.005

Table 5: Biochemical components were estimatedin housefly larvae against lemon grass oil.

Biochemical components	Lemon Grass		
	OD		
	1%	2%	3%
Protein (mg/gm Body Weight)	0.618 ± 0.019	0.518 ± 0.010	0.393 ± 0.010
Lipid (mg/gm Body Weight)	0.265 ± 0.019	0.150 ± 0.014	0.045 ± 0.006
Amylase (µmole/ml/min)	0.255 ± 0.019	0.175 ± 0.013	0.053 ± 0.010

Table 6: Biochemical components were estimatedin housefly larvae against Thyme oil.

Biochemical components	Thyme OIL		
	OD		
	1%	2%	3%
Protein (mg/gm Body Weight)	0.565 ± 0.019	0.458 ± 0.015	0.423 ± 0.030
Lipid (mg/gm Body Weight)	0.233 ± 0.010	0.148 ± 0.015	0.025 ± 0.010
Amylase (µmole/ml/min)	0.270 ± 0.012	0.175 ± 0.006	0.083 ± 0.005

5. Discussion

In larvicidal assay, it was found that essential oil was highly effective. It was also found that as the doses of each essential oil were increased the 1% mortality was also found. The mechanism of action of essential oils to insects is not clear but is reported to be diverse. In fact, it is difficult to make exact comparisons with other studies due to large variation in oil composition, target insect, mode/scale of experimentation, different exposure times and concentrations employed (Kumar *et al.*, 2011a).So it reflected by different affectivity of same oil in various assays. The increase in protein levels during larval development was due to synthesis of new proteins by the tissues, particularly fat bodies. There is a significant decrease in protein content of the 2nd instar larvae was observed in each test oils at different concentrations. The present results agree with the report of Martin, M.D (1990). He observed that during early stages of 2nd and 3rd instar larvae in *Calliphorastygia* there was a decrease in the soluble proteins and this decrease were correlated to low rate of protein synthesis by the fat bodies. Nagata and Kobayashi (1974) have also reported an increase in protein synthesis during feeding stage in *Musa domestica*. Hurlimann and Chen (1969) have reported that the concentration of protein in 2nd instar larvae increases progressively during larval development and reaches maximum in the late fifth instar larvae but declines in the pupal stage. The treatment of essential oils showed the decline of the protein content affects the normal synthesis of protein during larval development. The results of the present investigation also support the earlier stated report.

Thyme oil was also found to be effective in decrease the protein, lipid and amylase activities compared to the control group. Proteins occur naturally in all organisms. These biochemical components are involved in a multitude of physiological reactions from simple digestion of food to highly regulated cascades. The total protein and lipid was estimated in 1% (0.565 ± 0.019, 0.233 ± 0.010),2% (0.458 ± 0.015, 0.148 ± 0.015) and 3% (0.423 ± 0.030 and0.025 ± 0.010) of Thyme oil. Among that 3% showed a considerable effectiveness. Amylase is a digestive enzyme classified as a saccharidase (an enzyme that cleaves polysaccharides), which is made primarily by the hepatopancreas of crustaceans (Pan and Wang, 1997). The primary function of amylase is to break down starches in food (Hajen *et al*. 1993) and it is of great importance in nutrition intake. Moreover, the midgut has the highest amylase concentration and largest total amount of amylase of anyorgan in the housefly larvae (Von Borell, 2000). The amylase activity was estimated in 1% (0.270 ± 0.012), 2% (0.175 ± 0.006) and 3% (0.083 ± 0.005). The 3% could control the secretion of amylase was analyzed.

It has also been proposed that maximum growth rate of the housefly could partly beset by digestive capacity (e.g. proteolytic enzyme activity), oxygen availability, or the metaboliccapacity required to support tissue protein synthesis (Blier*et al.*, 1997). Chesson, (1987) stated that various

essential oils from herbs and spices are controlling the flies performance by inhibiting the action on gut secretions. The use of Citronella oil and other essential oils (e.g. Cinnamon) supplying cinnamaldehyde have been demonstrated to inhibit salivation (amylase production). The decrease in enzyme production can result in control of digestibility and availability of nutrients from feedstuffs (Chesson, 1987). The total protein and lipid was estimated in 1% (0.6375 ± 0.021, 0.43 ± 0.0231), 2% (0.5425 ± 0.030957, 0.3625 ± 0.0263) and 3% (0.435 ± 0.01and0.265 ± 0.0058) of Citronella oil treated with the 2nd instar larvae of *M. domestica.* The total protein and lipid content showed concentrations decreased from control (0.67 ± 0.012, 0.34 ± 0.014). The amylase activity was estimated in 1% (0.270 ± 0.012), 2% (0.175 ± 0.006) and 3% (0.083 ± 0.005), each concentration showed the decreased in concentration of amylase when compared to the control (0.348 ± 0.032). But, 3% could control and decrease the secretion of amylase, proved to be the efficient one inhibiting the digestive activities of amylase enzyme.

Insects utilize plant nutrients for their growth and development. Any concentration of the Ylangylang oil mixesd with the diet of the larvae results in adverse physiological changes. The environmental factors also have a direct impact on the morphological changes of the treated larvae (Hering and Taguchi, 1951). It was reported that the growth and development of housefly M. domestica and the morphological changes of their larvae were influenced to a great extent by the conception of protein and lipid contents from the diet (Krishnaswami et al., 1971). Moreover, the quantity, rate and quality of food consumed by an insect larvae has a great bearing on its survival, growth rate, developmental duration and final body weight (Ramadevi et al., 1993).The total protein and lipid was estimated in 1% (0.623 ± 0.013, 0.43 ± 0.0231), 2% (0.528 ±0.010, 0.245 ± 0.024) and 3% (0.475 ± 0.006 and0.138 ± 0.005) of Ylangylang oil treated with the 2nd instar larvae of *M. domestica*. The total protein and lipid content showed the concentrations were decreased when compare to control (0.67 ± 0.012, 0.34 ± 0.014). The amylase activity was estimated in 1% (0.345 ± 0.010), 2% (0.265 ± 0.010) and 3% (0.148 ± 0.005). Each concentration showed the decrease in concentration of amylase when compared to the control (0.348 ± 0.032). Increase in concentration of the oil leads to the gradual decrease in the amylase activity. The 3% concentration of the treated essential oil showed high level of control than the control between 1% and 2% were analysed. The protein contents of 2nd instar larvae treated with clove oil were lower when compared to control, which indicate the influence the of the oils (Rajkumar *et al.,* 2008). The variation of protein content could be attributed to difference in ecological distribution and temporal difference of the species contribute to the control of the biochemical components. The total protein and lipid was estimated in 1% (0.525 ± 0.0058, 0.245 ± 0.0192), 2% (0.4275 ± 0.0096, 0.14 ± 0.0142) and 3% (0.358 ± 0.015 and0.243 ± 0.0096) of Clove oil treated with the 2nd instar larvae of *M. domestica.* The total protein and lipid content showed the concentrations were decreased when compare to control (0.67 ± 0.012, 0.34 ± 0.014). The amylase activity was estimated in 1% (0.3225 ± 0.010), 2% (0.015 ± 0.006) and 3% (0.148 ± 0.005). Each concentration showed the decrease in the enzyme activity, reached its lowest values in larvae treated with 3% concentration of Clove oil when compared to the control (0.348 ± 0.032).

Lipids are highly efficient as sources of energy and they contain more than twice the energy of carbohydrates and proteins (Okuzumi and Fujii, 2000). The fatty acid content of diet modulates the fatty acid profile of tissue of the housefly larvae. This may be an effective way to inhibit the functionality of normal cells through diet treated with the lemon grass oil. Further, the lipid content differs greatly according to species, body part, season, habitat, feed and other factors. In the present study the total protein and lipid content was estimated in 1% (0.618 ± 0.019, 0.265 ± 0.019), 2% (0.518 ± 0.010, 0.150 ± 0.014) and 3% (0.393 ± 0.010 and0.045 ± 0.006) of lemon grass oil treated with the 2nd instar larvae of *M. domestica.* The total protein and lipid content showed the concentrations were decreased when compare to control (0.67 ± 0.012, 0.34 ± 0.014). The amylase activity was estimated in 1% (0.255 ± 0.019), 2% (0.175 ± 0.013) and 3% (0.053 ± 0.010). Each concentration showed the decrease in concentration of amylase when compared to the control (0.348 ± 0.032). Increase in concentration of the oil leads to the gradual decrease in the amylase activity. The 3% concentration of the treated essential oil showed high level of control than the control between 1% and 2% were analyzed. Lemon grass oil toxicity to different types of insects varies considerably. Other studies indicate that clove oil and lemon grass oil can be effective at controlling housefly at lower application rates.

To sum up, the present findings seem promising and therefore suggest that the usage of essential oils are more effective and may be considered as safer, eco-friendly and economic alternative to the synthetic insecticides. Such an approach would enable a reduced dose/concentration to be applied for a vector control program, thus potentially leading to improved resistance management and reduced costs. In addition, the present results may encourage housefly control research in the tropics using the essential oils.

6. Conclusion

In the present investigation the total protein, lipid and amylase contents levels are higher in 3% concentration when compared with that of 1% and 2% concentration of each essential oils. The total protein, lipid and amylase in the supernatant homogenated 2nd larval instar of *M. domestica* larvae was highly decreased as compared to control groups at all different concentrations. These changes indicate that various biochemicals specific changes occur at different concentration. The present findings seem promising and therefore suggest that the usage of essential oils are more effective to reduce the protein, lipid content and amylase activity in 3% concentration.

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