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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Anti-plasmodial and antioxidant activities of methanol extract of the fresh leaf of *Lophira lanceolata* (Ochnaceae)

Collins Azubuike Onyeto*, Peter Achunike Akah, Chukwuemeka Sylvester Nworu, Theophine Chinwuba Okoye, Nkemakolam Austine Okorie, Florence Nwakaego Mbaoji, Ifeoma Amarachukwu Nwabunike, Nelson Okumah and Obinna Okpara

Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria.

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This study was aimed at investigating the anti-plasmodial and antioxidant activities of the extract of the leaf of *Lophira lanceolata,* a traditional medicine recipe. The methanol extract (ME) obtained by 72 h cold maceration was evaluated for acute toxicity test (LD_{50}) and phytochemical constituents. The suppressive and curative anti-plasmodial activities of the extract were investigated using rodent malaria model. Mice (20 to 34 g) infected with 1×10^7 *Plasmodium berghei* parasitized red blood cell were used to test for suppressive and curative anti-plasmodial activities after oral administration of ME (100, 200 and 400 mg/kg) for four and seven days, respectively. The preliminary antioxidant activity of the extract (25, 50, 100, 200 and 400 µg/ml) was evaluated using *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, hydrogen peroxide scavenging and reducing (power) ability assays. The methanol leaf extract of *L. lanceolata* exhibited a dose-dependent suppression of parasitaemia up to 100% suppression at 400 mg/kg. The suppression produced by the extract was significantly ($P< 0.05$) higher than the chemo suppression produced by 20 mg/kg chloroquine (50.90%). Similarly, the extract at the same doses (100, 200 and 400 mg/kg) exhibited significant ($P<0.05$) but non-dose-related decreases in parasitaemia in the curative model (60.90, 54.69 and 79.69%) which was comparable to the 82.80% decrease caused by chloroquine (20 mg/kg). Phytochemical studies revealed the presence of flavonoids, alkaloids, oils, saponins, glycosides, carbohydrates, acidic compounds, terpenoids and reducing sugar. The LD_{50} test caused no deaths in the treated mice up to 5,000 mg/kg body weight. The DPPH assay, for free radical scavenging effect of the methanol extract was significant ($P<0.001$) as the concentration increases. Hydrogen peroxide scavenging and reducing power assays showed concentration-dependent and significant ($P<0.05$) results. These findings suggest that methanolic extract of the leaf of *L. lanceolata* is safe up to a dose of 5,000 mg/kg body weight and possesses anti-plasmodial and anti-oxidant activities.

Key words: *Lophira lanceolata*, anti-plasmodial, anti-oxidant, plasmodium berghei, DPPH, hydrogen peroxide, albino mice.

INTRODUCTION

Malaria, a global scourge remains a leading cause of morbidity and mortality worldwide, especially in pregnant women and children, and particularly in tropical Africa, where at least 90% of the malaria deaths occur (WHO,

2002). The disease kills about one million people globally each year, or about 3,000 people daily. About 40% of the world's population lives in malaria endemic areas, while nine out of every 10 malaria infection cases occur in sub-Saharan Africa. Despite significant progress in the treatment of malaria, it has staged a comeback in many areas of the world, due to the resistant by parasites (Najera, 2001; Shiff, 2002). Human malaria is caused by various species of plasmodia, with *Plasmodium falciparum, Plasmodium malariae, Plasmodium Ovale, Plasmodium vivax and Plasmodium Knowlesi (*Modupe et al., 2011) out of the 30 species, accounting for more than 95% of the cases of malaria in the world. *P. falciparum* is the most predominant parasite specie accounting for about 98% of malaria cases (Modupe et al., 2011). In Nigeria, malaria transmission occurs all-year round in the South, and is more seasonal in the North. The country accounts for a quarter of all malaria cases in the WHO African region (WHO, 2008). Free radicals, particularly reactive oxygen species (ROS) have a greater impact on humans. Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells to prevent damage to lipids, proteins, enzymes, carbohydrates of and DNA. Anti-oxidants play an important role in inhibiting and sca-venging radicals, thus providing protection against infections and degenerative diseases.

Lophira lanceolata (*Ochnaceae)* is widely distributed in the sudano-guinean savannah zone from Senegal through the Central African Republic and northernmost DR Congo to Uganda. Northeast Tropical Africa: Sudan; East Tropical Africa: Uganda; West Central Tropical Africa: Cameroon, Central African Republic and Zaire; West Tropical Africa: Benin, Gambia, Ghana, Guinea, Nigeria, Senegal, Sierra Leone, Cote d'Ivoire, Guinea-Bissau, Togo and Mali (Protabase). *L. lanceolata* is the commonest species in the dry savannah areas while *Lophira procera* is the species found in the forest zone of West Africa. Flowering is from December to February. It is a multipurpose tree; in traditional medicine meni oil is used to treat dermatosis, toothache and muscular tiredness. The sap of the tree is used to treat tiredness by the Dii, Fulbe and Gbaya peoples in Cameroon. In Mali pounded roots, mixed with flour are used to treat constipation, while its concoction is used to cure chronic wounds. A concoction prepared from the roots is drunk by women against menstrual pain, intestinal troubles and malaria. The bark of the roots and trunk is used against pulmonary diseases. The bark is also used to treat fevers and gastro-intestinal problems, and in southern Nigeria the root bark is a remedy for yellow fever. The young stems and sometimes the roots are commonly used as chew-sticks, and an infusion of the bark is used as a mouthwash against toothache in Guinea, Mali and Nigeria.

The present study was designed to investigate the antioxidant and antiplasmodial activities of *L. lanceolata* as acclaimed by the traditional medicine practitioners which might be useful to unravel novel treatment strategies for diseases associated with free radical induced tissue damage.

MATERIALS AND METHODS

Animals

Swiss albino mice (18 to 25 g) of either sex obtained from the Animal Facility centre of the Department of Pharmacology and Toxicology, University of Nigeria Nsukka, were used for the investigation. The animals were kept in cages at room temperature and naturally illuminated environment of 12:12 h dark/light cycle. They were fed on standard diet and had water *ad libitum*. Handling and use of animals were in accordance to the NIH Guidelines for the care and use of laboratory animals (NIH Publication No. 85 to 23, revised 1985).

Chemicals and instruments

1,1-diphenyl-2-picrylhydrazyl (DPPH) Methanol (analytical grade), Ferric chloride, potassium ferricyanide, ascorbic acid, hydrogen peroxide, trichloroacetic acid, phosphate buffer (pH 6.6 and 7.4) were all obtained from Sigma Chemical Company Ltd. (USA). Absorbance measurements were recorded by a Shimadzu UV-160A UV-Visible Reading Spectrophotometer (Shimadzu Corporation, Japan) using disposable cuvettes (Sarstedt, Nümbrecht, Germany) for visible range, and quartz cuvettes for measurements in the ultraviolet (UV) range.

Parasites

Parasitized erythrocytes were obtained from a donor-infected mouse maintained at Animal Facility Centre, Faculty of Veterinary Medicine, University of Nigeria Nsukka. Parasites were maintained by continuous re-infestation in mice. Animals were inoculated intraperitoneally with infected blood suspension (0.2 ml) containing 1×10^7 *P. berghei* parasitized red blood cell.

Plant collection and identification

The fresh leaves of the plant were collected in May, 2013 from Nsukka Enugu Stat, Nigeria. The plant was identified and authenticated by Mr Alfred Ozioko of International centre for Ethnomedicine and Drug development (Inter CED) Nsukka.

Preparation of plant extract

The leaves were air-dried at room temperature and ground into powder using a grinder (ADDIS, Nigeria). The powdered material

*Corresponding author. E-mail: kolinzuby@yahoo.co.uk. Tel: +234 803 400 4221.

(2370 g) was macerated with 4.5 L of 70 % methanol for 72 h with constant shaking. The resultant mixture was filtered using Whatman (No. 1) filter paper and the filtrate was concentrated to dryness in vacuum at 40°C using rotary evaporator. This gave a yield of 108.81 g (4.59% w/w).

Phytochemical test

Preliminary phytochemical studies were carried out on the extract for the presence of alkaloids, tannins, saponins, terpenes, flavonoids, oils, glycosides, steroids, and carbohydrates using standard procedures (Trease and Evans, 1989).

Acute toxicity test

The safety of the extract orally was evaluated by determining its LD_{50} using the Lorke's (1983) method. Dose levels used were from 10 to 5,000 mg/kg. All the animals were kept under the same condition and observed for signs of acute intoxication and mortality for 24 h. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose and the lowest lethal dose.

Experimental design and suppressive antiplasmodial assay

Evaluation of suppressive activity of the extract of *L. lanceolata* (4-days test) was performed as described by Knight and Peters (1980). Twenty five Swiss albino mice of either sex weighing (18 to 25 g) were inoculated by intra-peritoneal (i.p.) injection with 0.2 ml infected erythrocytes. The animals were divided into five groups of five per group and treated for four consecutive days. Group 1 and 2 received 3% Tween 80 (0.2 ml/kg) and chloroquine (20 mg/kg) daily, while groups 3, 4 and 5 received daily doses of the extract (100, 200 and 400 mg/kg), respectively. All administrations were by oral route. On day five of the study, thick and thin films were prepared with blood collected from the tail of each mouse. The films were fixed with methanol stained with Giemsa and parasitaemia was determined by counting the number of infected and uninfected red blood cells in 5 different fields. The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice.

Curative antiplasmodial assay

Evaluation of curative potential of aqueous methanol extract of *Lophira lanceolata* was done by adopting the method described by Ryley and Peters (1970). Twenty five mice were selected and intra-peritoneally injected with 1 x 10^7 *Plasmodium berghei* infected erythrocyte on the first day. 72 h after, the animals were divided into five groups of six per group. Group 1 received 3% Tween 80 (0.2 ml/kg) and chloroquine (20 mg/kg) daily by orally. While, group 3, 4 and 5 received daily doses of the extract orally (100, 200 and 400 mg mg/kg, respectively). Treatment continued until the fifth day when thick and thin films were prepared with blood collected from the tail of each mouse. The films were fixed with methanol, stained with Giemsa and parasitaemia was determined by microscopic examination in 5 different fields. The mean survival time for each group was determined by finding the average survival time (days) of the mice in each group was calculated.

In vitro antioxidant tests

Tests on reducing power

The reducing power of the extracts was evaluated as described by Oyaizu (1986). Briefly, 1 ml of the test sample is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6), 2.5 ml of potassium ferricyanide (30 mM) and incubated in a water bath at 50°C for 20 min. Trichloroacetic acid solution (2.5 ml: 600 mM) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was carefully removed and combined with 2.5 ml of distilled water and 0.5 ml of 5 mM ferric chloride and the absorbance of the reaction mixture were measured at 700 nm. Ascorbic acid diluted in methanol was used as a standard.

Hydrogen peroxide scavenging assay

The ability of the *L. lanceolata* extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989). A solution of hydrogen peroxide (43 mM) was prepared in phosphate buffer (pH 7.4). Different concentration of extracts (25, 50, 100, 200, 400 µg/ml) were added to a hydrogen peroxide solution (1 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenged by both extract and standard compounds were calculated.

DPPH radical scavenging assay

DPPH radical scavenging activity was measured using the method of Blois (1958). The extract (1 ml) was added to 1.0 ml of DPPH in methanol (0.3 mM) and mixed with 1.0 ml of 5 mM DPPH in methanol. The reaction mixture was then kept in dark at room temperature for 10 min. Positive control used was ascorbic acid. The absorbance of the resulting solution was measured at 517 nm. The decrease in absorbance at 517 nm was calculated as the percentage of inhibition using the following equation.

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

A_0 = absorbance of control, A_1 = absorbance of the tested sample.

Statistical analysis

Results were expressed as mean ± S.E.M. The data was analyzed using Student's t-test and one way ANOVA: LSD post hoc compared to control using SPSS version 16 software. $P<0.05$ and $P<0.001$ were considered statistically significant.

RESULTS

Phytochemical analysis

In the preliminary phytochemical screening, the methanol leaf extract of *L. lanceolata* gave positive test for flavonoids, alkaloids, oils, saponins, glycosides, carbohydrates, acidic compounds, terpenoids and reducing sugars.

Acute toxicity

In the acute toxicity test, no death was recorded even up to 5,000 mg/kg; hence the LD_{50} of the *L. lanceolata* is above 5 g/kg.

Table 1. Suppressive effect of methanol leaf extract of *L. lanceolata* against *P. berghei* in mice.

Treatment	Dose (mg/kg)	Parasitaemia count	% Inhibition
Control	2 ml/kg	11.0 ± 3.27	
Lophira lanceolata	100	2.60 ± 1.96	23.63
Lophira lanceolata	200	5.80 ± 1.96*	52.72
Lophira lanceolata	400	8.80 ± 1.96*	80.00
Chloroquine	20	5.60 ± 1.96*	50.91

Results are Mean count ± S.E.M. (n = 5). * $P < 0.05$.

Table 2. Curative effect of methanol leaf extract of *L. lanceolata* against *P. berghei* in mice.

Treatment	Dose (mg/kg)	Parasitaemia count	% Inhibition
Control	2 ml/kg	12.8 ± 1.24	
Lophira lanceolata	100	4.6 ± 0.87*	64.06
Lophira lanceolata	200	5.8 ± 1.43*	54.69
Lophira lanceolata	400	2.6 ± 0.51*	79.69
Chloroquine	20	2.2 ± 0.49*	82.81

Results are Mean count ± S.E.M. (n = 5). * $P < 0.05$.

Table 3. Reducing power effect of methanol leaf extract of *L. lanceolata*.

Concentration (µg/ml)	Absorbance (700 nm)	
	Extract	Ascorbic acid
25	0.7690 ± 0.0015*	0.2530 ± 0.0006
50	0.8597 ± 0.0015*	0.2660 ± 0.0000
100	1.0183 ± 0.0007*	0.2847 ± 0.0003
200	1.0167 ± 0.0015*	0.2807 ± 0.0003
400	1.1120 ± 0.0040*	0.3673 ± 0.0007

Values are represented as Mean ± S.E.M (n=6); *$P<0.05$

Antiplasmodial activities

Suppressive effect

The methanol leaf extract of *L. lanceolata* exhibited a dose-dependent suppression of parasitaemia with 100% suppression at 400 mg/kg. The suppression produced by the extract was significantly ($P< 0.05$) higher than that produced by chloroquine 50.90% (Table 1).

Curative effect

The methanol extract, at the same doses (100, 200 and 400 mg/kg) exhibited significant ($P<0.05$) but non-dose-related decrease in parasitaemia (60.90, 54.69 and 79.69%) which was comparable to that of chloroquine (20 mg/kg, 82.80%) (Table 2).

Antioxidant assays

Test on reducing power

The reducing power of ascorbic acid and methanol extract increased in a concentration-dependent manner. The extract exhibited a significantly ($P< 0.05$) higher reducing power activity than ascorbic acid used as standard at same concentrations (25, 50, 100, 200 and 400 µg/ml). The highest reducing power was seen at 400 µg/ml of the extract (1.1120±0.0040) compared to the standard, ascorbic acid at same concentration (0.3673±0.0007) (Table 3 and Figure 1).

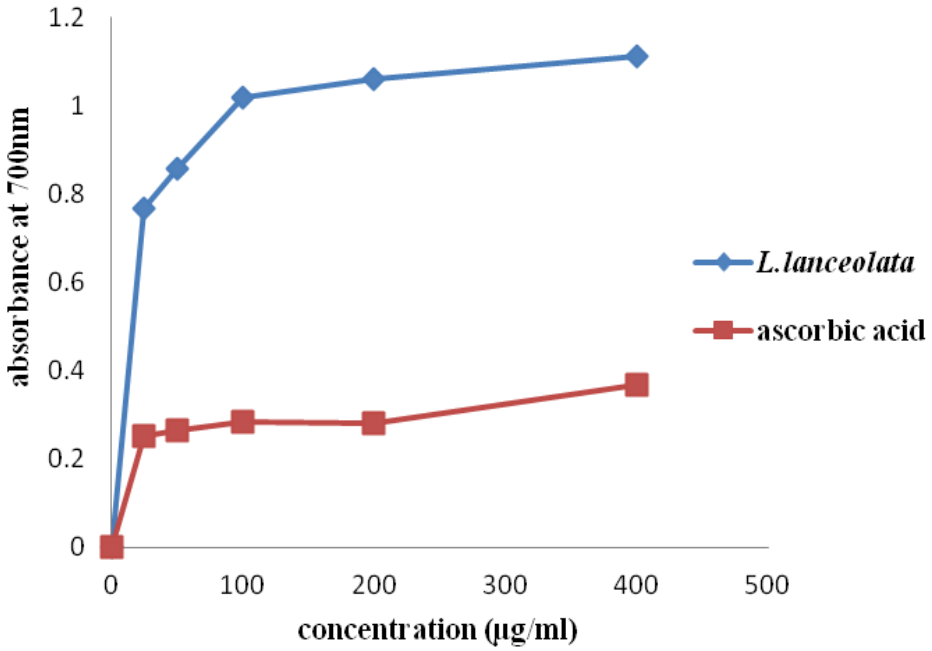

Figure 1. Reducing power ability of *L. lanceolata* extract and ascorbic acid at various concentrations.

Table 4 DPPH Radical Scavenging effect of *L. lanceolata.*

Conc. (µg/ml)	Absorbance (517 nm)		% Inhibition	
	Extract	Ascorbic acid	Extract	Ascorbic acid
25	0.2010±0.0020*	0.2025±0.0005*	87.14	87.14
50	0.1250±0.0100*	0.1860±0.0010*	92.00	88.11
100	0.1045±0.0250*	0.1825±0.0005*	93.31	88.33
200	0.0895±0.0035*	0.1680±0.0010*	94.27	89.25
400	0.0870±0.0010*	0.1660±0.0010*	94.43	89.38
Control	1.5640±0.0057			

Values are represented as Mean ± S.E.M (n=6); *P<0.001.

DPPH radical scavenging assay

DPPH radical scavenging activity of methanol leaf extract of *L. lanceolata* was compared with ascorbic acid. The extract exhibited a significantly (P< 0.001) higher DPPH radical scavenging activity compared to the standard; ascorbic acid at the same concentrations (25, 50, 100, 200 and 400 µg/ml) in a dose dependent manner. At concentration of 400 µg/ml, the DPPH radical scavenging activity of the extract was found to be 94.43% and that of ascorbic acid was 89.38% (Table 4 and Figure 2).

Hydrogen peroxide scavenging assay

The free radical scavenging activity of the methanol leaf extract of *L. lanceolata* showed significant (P<0.001) concentration dependent activity with the hydrogen peroxide scavenging effect of the extract of 400 µg/ml (95.19%) higher than that of ascorbic acid 400 µg/ml (88.01%) used as standard (Table 5 and Figure 3).

DISCUSSION

The results indicate that the methanol leaf extract of *L. lanceolata* possessed antiplasmodial activity as shown in the significant chemo suppression data obtained from the early and established infection. In the acute toxicity test there was no deaths in mice within 24 h up to 5000 mg/kg oral dose. This suggest that the extract is relatively nontoxic acutely. The chemosuppression obtained in the

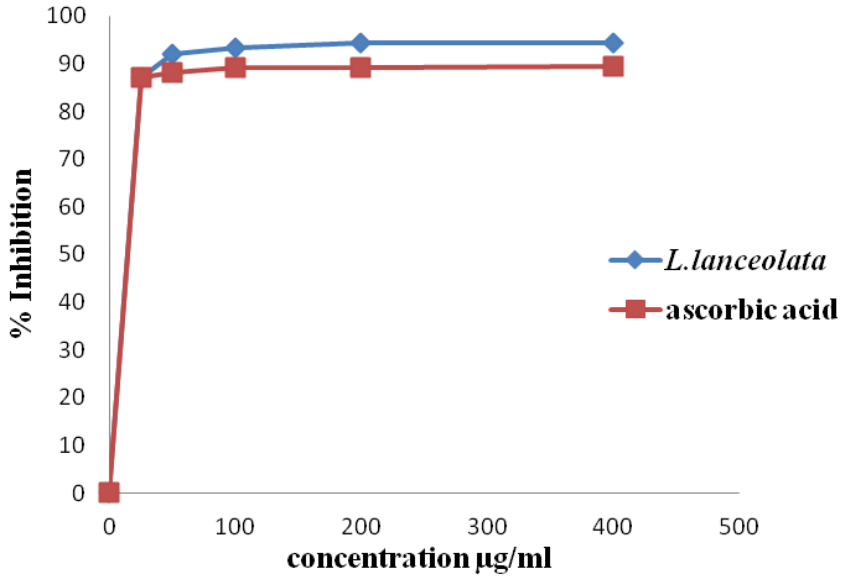

Figure 2. DPPH Radical scavenging activity of methanol extract of *L. lanceolata.*

Table 5 Hydrogen peroxide scavenging effect of *L. lanceolata*

Concentration (µg/ml)	Absorbance (230 nm)		% Inhibition	
	Extract	Ascorbic acid	Extract	Ascorbic acid
25	0.0670±0.0006*	0.1980±0.0005*	93.47	80.70
50	0.0657±0.0007*	0.1697±0.0010*	93.56	83.53
100	0.0657±0.0003*	0.1540±0.0005*	93.56	84.99
200	0.0543±0.0003*	0.1347±0.0010*	94.74	86.84
400	0.0510±0.0006*	0.1233±0.0010*	95.19	88.01
Control	1.0263±0.0003			

Values are represented as mean ± S.E.M (n=6); *P<0.001.

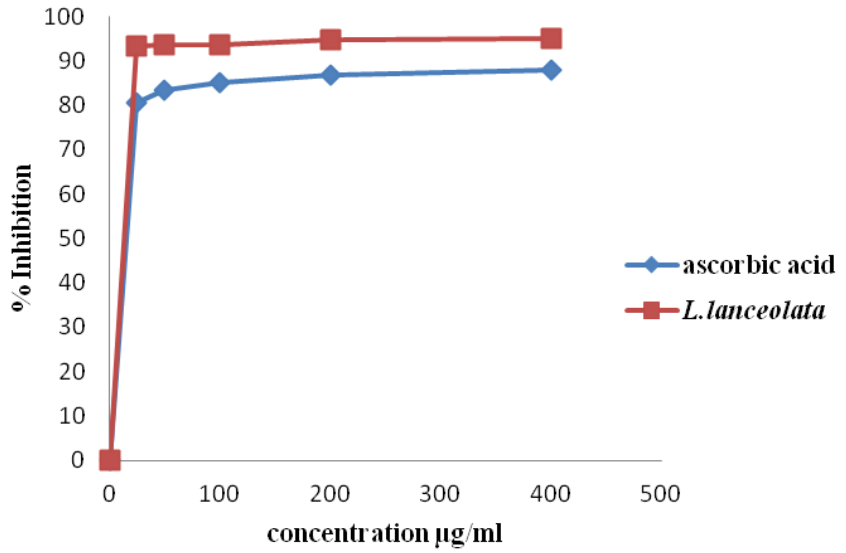

Figure 3. Hydrogen peroxide radical scavenging of *L. lanceolata* compared to ascorbic acid at various concentrations.

4-day early test (suppressive) was dose dependent comparable to the standard drug; chloroquine. Agents with suppressive activity against *P. berghei* were known for antimalarial activity, (Calvalho et al., 1991). Also, chemo suppression of parasitaemia recorded in established infection (curative) was comparable to the standard drug; chloroquine but not dose dependent. The chemosuppression of parasitaemia seen in early infection (suppressive) exhibited a dose-dependent suppression of parasitaemia with 100% suppression at 400 mg/kg which was significantly ($P< 0.05$) higher than that produced by chloroquine 50.90%. Similarly, the methanol extract at the same doses (100, 200 and 400 mg/kg) exhibited significant ($P<0.05$) but non-dose-related decreases in parasitaemia (60.90, 54.69 and 79.69%) recorded in established infection (curative) which was comparable to that of chloroquine (20 mg/kg, 82.80%).

The mechanism of action of the extract for antiplasmodial activity could be either by causing red blood cell oxidation (Etkin, 1997) or by inhibiting protein synthesis (Kirby et al., 1989) depending on their phytochemical constituents. The extract could have exerted its action through either of the two mechanisms mentioned above or by some other unknown mechanism. There are numerous antioxidant methods for evaluating of antioxidant activity. For *in vitro* antioxidant screening, (DPPH) free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging and ferric thiocyanate reducing activities are most commonly used. However, the total antioxidant activity of an antioxidant cannot be evaluated by using one single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity (Ilhami et al., 2005). The decrease in absorbance of DPPH radical caused by methanol leaf extract of *L. lanceolata* was due to the reaction between antioxidant molecule and radical which results in the scavenging of the radical by hydrogen donation (Soares et al., 1997). It is visually noticeable as a discoloration from purple to yellow in addition of the extract in a concentration-dependent manner. However, Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Nabavi et al., 2009). In the reducing power assay for the measurements of the reductive ability the Fe^{3+} to Fe^{2+} transformation in the presence of the methanol extract was investigated and found to have significant reducing ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

The methanol leaf extract of *L. Lanceolata* showed moderate scavenging effects. As shown in Figure 3, the extract demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner. Scavenging activity of H_2O_2 by the extract may be attributed to their phenolic content which can donate electrons to H_2O_2 thereby neutralizing it into water (Mathew and Abraham, 2006). Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It rapidly transverses cell membrane and once inside the cell interior, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Contreras-Guzman and Strong, 1982). Thus, the removal of H_2O_2 is very important for antioxidant defence in cell or food systems. H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results showed that *L. lanceolata* has an effective H_2O_2 scavenging activity.

The antioxidant activity of compound has been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Phytochemical analysis revealed the presence of anthroquinones, flavonoids, carbohydrates, oils glycosides, phenols, saponins, steroids, tannins and free reducing sugar. Generally, flavonoids are the important class of antioxidants; hence the medicinal plants containing flavonoids and phenolic compounds are repeatedly screened for antioxidant activity. In addition to flavonoids and phenolic compounds, some of the alkaloids, saponins and triterpenoids are reported to possess antioxidant activity (Rai et al., 2006). The presence of flavonoids, alkaloids and terpenoids in *L. lanceolata* has been reported (Audu et al., 2007) and the results of the phytochemical investigation in the present study also further substantiated this. Hence, the results suggest that the methanol leaf extract of *L. Lanceolata's* antioxidant activity may be due to the presence of flavonoids and tannins and the observed *in vitro* antioxidant activity may be because of these phytoconstituents.

Conclusion

The outcome of this investigation revealed that, the methanol leaf extract of *L. lanceolata* is safe and possess potent antimalarial and antioxidant activity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Review

Genetic transformation of lettuce (*Lactuca sativa*): A review

Song Dan, Han Qiang, Dong Zhaonan and He Zhengquan*

Biotechnology Research Center of China Three Gorges University, Yichang Hubei 443002, China.

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Lettuce (*Lactuca sativa* L.) is a globally important leafy vegetable that can be grown worldwide. Due to the rapid growth of population and the human desire to progress, there have been a lot of studies made by researchers, especially in genetic engineering. Improvements in regeneration system and transformation methodology have helped to increase the transformation efficiency and stable expression of transgenes in lettuce. Lettuce transgenic research carried out so far has mainly focused on using lettuce bioreactor to produce pharmaceutical protein and vaccines, improving nutritional and physiological value of lettuce. There are no comprehensive and detailed reviews available combining research developments with major regeneration system and basic genetic transformation in lettuce. This is an attempt to overview the progress in regeneration system, genetic transformation and biotechnological applications in the last decades as well as future implications.

Key words: Lettuce, regeneration system, genetic transformation, bioreactor.

INTRODUCTION

Lettuce (*Lactuca sativa L.*) belongs to the Asteraceae family, one of the major crops grown wordwide. The plants often have a height of 15 to 30 cm, with colorful leaves running from bright green to red and yellow. Lettuce also have a wide range of shapes and textures, from the dense heads of the iceberg type to the notched and scalloped (Martha, 2011). It is low in calories, good-tasted and nutritive, which is a good source of vitamin A, vitamin K and potassium to the human population. Its stems and leaves contain many active

*Corresponding author. E-mail: zhq_he@163.com. Tel (fax): +86-717-6397188.

Abbreviations: **2,4-D**, 2,4-Dichlorophenoxyacetic acid; **6-BA**, 6-benzylaminopurine; **hpt**, hygromycin B phosphotransferase; **HPLC-ELSD**, high performance liquid chromatography-evaporation light-scattering detection; **IAA**, indole-3-acetic acid; **NAA**, 1-naphthyleneacetic acid; **nptII**, neomycin phosphor-transferase II gene; **PPT**, phosphinothricin; **AI**, avian Influenza; **A.T.**, *Agrobacterium* tumefaciens-mediated transformation; **ChIFN-α**, chicken alpha interferon-α; **DAS-ELISA**, double Antibody Sandwich-Enzyme Linked Immunosorbent Assay; **ELISA**, enzyme linked immunosorbent Assay; **FMD**, foot-and-mouth disease; **GUS**, glucuronidase; **HBV**, hepatitis B virus; **HIV**, human immunodeficiency virus; **HPLC**, high performance liquid chromatography; **Human IFN**, human interferon; **Hyg**, hygromycin; **IL-2**, interleukin-2; **Km**, kanamycin; **Pb**, projectile bombardment; **PCR**, polymerase chain reaction; **PPT**, phosphinothricin; **RT-PCR**, reverse transcription polymerase chain reaction; **sCT**, salmon calcitonin; **SARS**, severe acute respiratory syndrome; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **Spec**, spectinomycin.

ingredients such as mannitol (which take effects on diuretic and blood circulation promotion) and lactucerin (which play a role on hypnosis, analgesia, and adjuvant treatment of neurasthenia). Nowadays, extracts from *Lactuca sativa L.* have been used for curing sunburn and rough skin in creams and latexes (Odu and Okomuda, 2013).

However, plant diseases and insect pests are standing out in the cultivation and production of lettuce. Plant diseases seriously affect the quality and yield of lettuce. For example, *Lactuca sativa Sclerotinia* has occurred in the world of lettuce, which seriously harmed the basal part of stems and leaves (Waipara, 2006; Chitrampalam et al., 2010). So many experts committed themselves to studying transgenic lettuce. Various types of genes which were transferred into lettuces, being expressed and stably inherited in progenies, are summarized in Table 1.

Lettuce used as a foreign protein expression system has the following advantages (Guo, 2006):

i) Exogenous gene expression product could be successfully post transcriptionally processed and modified with low cost and is relatively safe,
ii) Lettuce is a well-loved vegetable that can be eaten directly. So lettuces with special functions could be used for the prevention or treatment of diseases, relieve the patients' spiritual and economic pressure as much as possible,
iii) Lettuce resistance to cold ecological environment can be cultivated over a wider area, easy to scale and are grown almost all over the world,
iv) The genetic transformation technology of lettuce is relatively mature, which provides favorable conditions for the use of lettuce to express foreign protein.
v) As a plant bioreactor, lettuce's production cycle is shorter than a lot of plant, the production superiority of fast speed, simple condition and low cost will stand out if used in a large scale of producing some protein or vaccine.

REGENERATION SYSTEM

An efficient and stable regeneration system is the basis of most genetic transformation technologies such as *Agro-bacterium* mediated and micro-projectile bombardment transformation. The tissue culture of lettuce starts early in lettuce and organogenesis has been the extensively used pathway compared to somatic embryogenesis for its wider adaptability among diverse genotypes. Protocols for obtaining stable regeneration in lettuce have been reported through organogenesis from callus (Gao, 2003), differentiated non meristematic tissues like leaf (Gao et al., 2011; Liu et al., 2011) and various seedling explants such as hypocotyls (Gao et al., 2002), cotyledons (Luo et al., 2010; Chen et al., 2012). A suitable transformation regeneration system should have adequate source of explants, genetic stability of the regenerated plants, great regeneration ability, sensitive to *Agro-bacterium* or other conversion, and a modest antibiotic sensitivity. In the establishment of lettuce regeneration system, genotype, different hormone groupings, explant type, seedling age and other factors have been studied by researchers. Although some reports have shown a higher frequency of regeneration, materials or other reasons lead to poor reproducibility, this article reviews the relevant factors needed to accelerate and facilitate the application of this technique in lettuce transformation.

GENOTYPE/CULTIVAR

It is well known that genotype has been the major factor which can significantly influence the regenerative capacity of explants. As in many other crops, shoot production in lettuce is also genotype-dependent. The frequency of shoot regeneration from cotyledon explants ranges from 84.6 to 100% of 4 lettuce species (Zhu et al., 2002). Explants of 5 cultivars were cultured under similar culture conditions, but only two lettuce cultivars (TN-96-39, TN-96-41) showed the best callus production, embryogenesis, regeneration and proliferation (Honari et al., 2008). The regeneration response in similar culture media showed big variability from cotyledon explants between the genotypes, differential frequency of regeneration ranging from 51 to 96% of 15 lettuce cultivars (Denise et al., 2002). Such variations have also been reported for many other genotypes. Genotype dependency for shoot regeneration has also been evidenced from leaf explants of *Lactuca sativa* L. cv. America Head Garden, America Violet Leaf and America Big Leaf Hydrangea (Liu et al., 2011). The effect genotype has on a species capacity to respond to shoot production has been recognized, so select an appropriate genotype is considerably important on the smoothness of studying genetic transformation.

EXPLANT TISSUE

Differential sources of explants have been used for the induction of shoots in lettuce, including leaf, hypocotyl, root, stem, cotyledon and cotyledon petiole. By using same genotype; a regeneration frequency of 65，85 and 95% was achieved through hypocotyl, cotyledon and cotyledon petiole, respectively, and the shoots in cotyledon and hypocotyl explants failed to respond greater than cotyledon (Li, 2007). Similar results were obtained with explants such as leaf, stem and root in Grand Rapids, meanwhile the rate of callus induction and bud generation from explants of leaves were significantly better than root and stem explants (Gao et al., 2011). However, an opposite conclusion appeared that the time and number of buds germination of cotyledon explants

Table 1. Genetic transformation of lettuce.

Genotype	Method	Detection	Selection	Plasmid vector	Function	Reference
Grand rapids	A.T.	PCR, PCR-Southern, RT-PCR	Hyg	pCAMBIA1301	FMD	Deng et al. (2007)
VitoriadeVerao	A.T.	PCR	Km	PG35SHBsAg	HBV	Jackson and Ekkehard (2008)
Glass lettuce	A.T.	PCR, RT-PCR	Km	pBI121-NA	AI	Fan et al. (2012)
Beijing lettuce	A.T.	PCR, PCR-Southern	Km	pEbislycBHyg, pBI121-gg	HIV	Jing et al. (2007)
Lactuca sativa	A.T.	PCR, Northern blot, Immunoblot, GM_1-ELISA	Km	pMYV514	Cholera toxin	Huy et al. (2011)
Simpson elite	Pb	Southern blot, western blot, GM_1 BA, histochemistry, immunohistochemistry	Spec	pLS-LF-CTB-Pins	Cholera toxin	Tracey et al. (2007)
Snezhinka et al.	A.T.	PCR, RT-PCR	Km	pCB063, pCB064	Tuberculosis	Matvieieva et al. (2009)
Green Wave	A.T.	PCR, Southern blot, RT-PCR, ELISA, Western blot, Test animal trails	Km	pBIF-V	Plague	Sergio et al. (2010)
Lactuca sativa	A.T.	PCR, Northern blot, Western blot, Laser-Scanning lonfocal Microscopy	Spec	pCV1, pCV2, pCV12	SARS	Li et al. (2006)
Lactuca sativa	A.T.	PCR, Northern blot, Immune blot, ELISA, GM_1-ELISA	Km	pMYO51	Heat-labile enterotoxin	Kim et al. (2007)
Lactuca sativa	A.T.	PCR, Immune blot, ELISA, Western blot, FAS, Mouse feeding assay	Km	pBI121-*espA*	Enterohaemorrhagic *E. coli*	Luan et al. (2009)
Japanese soft glassy et al.	A.T.	GUS assay, PCR, SDS-PAGE, Western blot, Antiviral activity assay	Km	pBI121-IFN	Human IFN	Li et al. (2007)
America Grand rapids et al.	A.T.	PCR, RT-PCR, HPLC, GC-MS	Km	pBI-RS	ResveratrolSynthase	Zhu, (2008)
Zhouye	A.T.	PCR, Southern blot, RT-PCR	Km	p35S-2300-twinT-DNA::pil-msCT::noster	sCT	Cui et al. (2009)
Grand rapids	A.T.	PCR, ELISA, Differential spectroscopy	Km	pGBIVHbTα1hLf, pGBIRVHbTα1hLf	Human lactoferrin and Thymosin	Meng et al. (2005)
Lactuca sativa	A.T.	PCR, RT-PCR	Km	pBI-121-NK	nattokinase	Tian, (2007)
Lactuca sativa	A.T.	PCR, Southern blot, ELISA, Western blot	PPT, Km	-	IL-2	Yang et al. (2008)
Lactuca sativa	A.T.	GUS assay, RT-PCR, ELISA	Km	pSFIFN-α	ChIFN-α	Song et al. (2008)
Italian Yearly Late Bolt	A.T.	PCR, RT-PCR，HPLC-ELSD	Km	p2301-GMP-myc	Vitamin C	Wang et al. (2011)
*longifolia*Lam.	A.T.	PCR, RT-PCR，HPLC	Km	pCAMBIA2300-35S::LsHPT::NOS	Vitamin E	Ren et al. (2011)
Grand rapids	A.T.	PCR, Southern blot, RT-PCR，AA analysis	Km	pBI121-lrp	Lysine	Li et al. (2006)
Romaine	A.T.	RT-PCR, HPLC, *Lactobacillus casei*fermentation	Km	pFSndt5100-*Atpsy -folE*	Carotenoids and Folic Acid	Fu et al. (2012)

Table 1. Contd.

Huaxuan No. 1	A.T.	PCR, Southern blot, RT-PCR, Nematode，Histological Analysis	Km	pBI121	Resistance to Root Knot Nematodes	Li et al. (2010)
Evola	A.T.	PCR, RT-PCR	Km	pBIPTA	Resistance to Aphids	Ahmed et al. 2007
Kaiser	A.T.	DAS-ELISA, Western blot, Southern blot, Northern blot	Km	pYK23	Resistance to *Mirafiori* virus	Yoichi et al. (2009)
Verônica	A.T.	PCR，RT-PCR	Hyg	pCambiaOxDc	Resistance to *Sclerotiniasclerotiorum*	Dias et al. (2006)
Chongchima	A.T.	Histochemical GUS staining, Southern blot, Northern blot, Drought and cold stress tests	Hyg	pCUMB	Resistance to drought and cold	Enkhchimeg et al.(2005)

were markedly better than leaf (Zhu et al., 2002).

These two opposite results demonstrated that genotype also influenced the induction of different explants tissue. Song et al. (2007) found a holographic phenomenon when using different part of cotyledon explants, the rate of bud induction in full cotyledon with or without petiole was higher than in the half and one-third of cotyledon. In addition, the rate of adventitious buds induction of integrated leaves were lower than the cotyledon petiole, which might be because the petiole had two incisions for absorbing more nutritional ingredients and hormone (Song et al., 2007). Although the explants diversification has been exploited and displayed a greater regeneration capacity, the leaf explants with callus-mediation had a greater potential in transformation through *Agrobacterium* as well as DNA bombardment (Gaurav et al., 2010).

SEEDLING AGE

In the choice of seedling age, it is generally believed that cotyledon explants from seedlings 2-4 days after germination are in favor of adventitious bud regeneration. Shoots induction and callus regeneration is quite different in different seedling ages and different types. The researchers also showed that 2-3d cotyledon explants just launched was the best in bud differentiation, even up to 100%. Similar results also appeared in the experiment of Chen et al., who compared the adventitious buds of Italy lettuce cotyledons differentiation rate of 3-4 days with 7-10; 3-4days lettuce cotyledons was significantly higher than the 7-10 days lettuce cotyledons (Chen et al., 2012). This may be because of the actively physiological metabolism of small seedling age cotyledons is vulnerable to the impact of external factors such as exogenous hormones. To the same organ and tissue, it is easier to be cultivated and regenerated for the juvenile state explants than adulthood plants.

However, to our surprise, for some species cotyledon age is not a barrier to efficient shoot regeneration. The cultivars Great Lakes, Greenway et al. were unaffected by cotyledon age, with no significant difference in the mean number of shoots produced for cotyledons excised 3–14 days after germination(Denise et al., 2002). In contrast, shoot production from explants of the other cultivars tested, showed a reduced ability to produce shoots as cotyledon age increased. To our knowledge this is the first report of a genotype dependent effect of explants age in lettuce. This might account for the considerable variation in the percentage of explants forming shoots when previously published studies are compared (Denise et al., 2002).

MEDIA COMPOSITION AND PLANT GROWTH REGULATORS FOR LETTUCE REGENERATION SYSTEM

MS and 1/2 MS medium have always been used for lettuce media composition. Plant growth regulators play a primary role in growth regulation rather than nutritional supplementation in plant regeneration and development (Slater et al., 2003). Plant growth regulators in different concentrations have obvious effect not only on callus explants tissues formation but also on shoot regeneration. Four growth regulators: NAA, IAA, 6-BA and 2, 4-D which are very familiar to us have been used in varied concentrations and combinations for shoot regeneration in tissue culture. Regeneration of seven shoots from cotyledon explants were obtained on MS medium enriched with 6-BA (0.1 mgL-1) and NAA (0.05mgL^{-1}) (Liu et al., 1996). While at a high concentration of 6-BA (1.2 mgL^{-1}), a similar number of shoots were obtained (Song et al., 2007). In contrast, MS medium supplemented with a high concentration of BA (0.44μM) did not increase the number of shoots produced per explants, but inhibited shoot development allowing

callus to proliferate (Denise et al., 2002). The combination of auxin like NAA and IAA has been extensively used in many composite crops resulting in advanced shoot initiation and producing a maximum number of shoots from explant of cotyledon. The result of Deng et al. demonstrated that 0.2 mgL^{-1} IAA in combination with 1.5 mg L^{-1}6-BA in the MS medium stimulated callus formation and improved the percentage of explants that regenerated (Deng et al., 2007). Successful rooting of regenerated shoots was not only achieved on 1/2 MS medium but also supplemented with NAA and IAA, and NAA was also found to be more effective than IAA in many reports (Zhu et al., 2002; Chen et al., 2012).

The species of Zhengyuan Italy lettuce were selected and the regeneration system was optimized in my work. Cotyledons as the explants were prepared by cutting of 5-day-old seedling and including 2-day-old cultured in the dark, and then transferred to the adventitious buds differentiation medium MS+0.5mgL^{-1} 6-BA+0.3 mgL^{-1} NAA with faster and better growing. The shoot regeneration rates were generally high, which was similar to the above research.

ADVANCES IN GENETIC TRANSFORMATION

Genetic transformation is an important tool in addressing increasing worldwide demands for lettuce with more academic and agronomic value. A variety of methods now exist for lettuce genetic transformation. The most frequently used methods are *Agro-bacterium* mediated transformation and particle bombardment, with the former having a much higher transformation frequency and efficiency, and the latter breaking the limitations of carrier method. Of the transgenic lettuce experiments described to date, the majority of them focus on three special areas critical to lettuce: pharmaceutical protein, vaccines, and nutritional value. Recent advances in the genetic transformation of lettuce have made it possible to transfer various chimeric genes of pharmaceutical and nutritional importance to the genome of recipient species. It is presumed that this technology may help to make up for some of the limitations of classical breeding associated with lettuce improvement (Fu et al., 2007).

TRANSGENIC LETTUCE TO PRODUCE VACCINE

The production of vaccine in transgenic plants is one of the hot spots of vaccine development nowadays. Oral vaccination, as a novel vaccine molecules expression system, could eliminate the economic burden and pain of injection and possess unparalleled advantages. These convenience factors could lead to better compliance for patients, both in developing and developed countries. In recent years, the ability of transgenic plants to induce an immune response via oral route has gradually been confirmed, it is not only capable of expressing exogenous vaccine protein but also stimulate the effective protection of mucosal immune and system immune (Li and Xi, 2004). A lot of plant vaccines were successfully expressed in lettuce, including hepatitis B vaccine (Jackson et al. 2008), foot and mouth disease vaccine (Deng et al. 2007), avian influenza vaccine (Fan et al., 2012), et al, among which hepatitis B vaccine expressed in lettuce was frequently studied by researchers. It was confirmed that most of the transformation resistant lettuce plants detected by PCR, PCR-Southern and other molecular analysis grow very well, which laid the foundation of lettuce as a bioreactor to produce vaccine. In most cases, marker genes that confer antibiotic resistance such as neomycin phosphotransferase (*npt*Ⅱ) and Hygromycin B-phosphotransferase (*hpt*) have been used in lettuce resistance selection.

Transgenic lettuce plant could exhibit higher protein accumulation, indicating that increased mRNA level in transgenic plants contributed to increased protein levels. It was important to decrease the feeding amount during immunization due to the high expression of vaccine antigen in transgenic plants. The low expression of antigen gene in transgenic plants was problematic to efficient induction of immune responses. To test the feasibility of oral vaccine, five-week-old mice were used in study to demonstrate the alleviation of symptomatic pancreatic and the preservation of insulin-producing β-cells. This was the first report of expression of therapeutic protein in transgenic chloroplasts of lettuce (Tracey et al., 2007). On the basis of the results obtained in previous study, human clinical trials would have been initiated, which will open up the possibility for the low-cost production and delivery of human vaccines, and a strategy for the treatment of various other autoimmune diseases (Tracey et al., 2007).

TRANSGENIC LETTUCE TO PRODUCE PHARMACEUTICAL PROTEIN

The production of pharmaceutical competent proteins and peptides in plants was another rapidly developing area in the application of transgenic plants in recent years. While the choice of an efficient expression system for production of a therapeutic protein or peptide was influenced by several factors like technical and economical. Numerous studies showed that human with little content of important clinical value of protein or polypeptide could be expressed in the plant system. Many proteins such as peptide hormones, insulin and interferon have been successfully expressed, in which peptide hormones have been studied more often, mainly calcitonin (Cui et al., 2009) and thymosin (Meng et al., 2005). The initial question to be solved was whether a given expression system could produce these proteins in an active form that could be administered to the patients. However, the low level of

expression was still the main problem facing in current production practice, so the expression in lettuce still needed to be substantially improved and its genetic stability to be further investigated.

In the present study, *Agro-bacterium* mediated system has previously been examined to validate the expression of Human lactoferrin and Thymosin (Meng et al., 2005) or produce some nattokinase (Tian, 2007). ChIFN-α was correctly transcribed and expressed in lettuce plants, and the recombinant IFN obtained was active for conferring protection against VSV infection (Song et al., 2008). These findings could be valuable for prevention of many cardiovascular and cerebrovascular diseases and neoplastic diseases in old people.

GOOD QUALITY RESEARCH OF TRANSGENIC LETTUCE

Lettuce is a type of leafy vegetable rich in vitamin. The nutritional value of its transgenic research was currently focused on the increase of content of vitamin A, vitamin C and vitamin E, and followed by the resistance of lettuce. Wang et al. transferred the gene-containing GDP-mannose pyrophosphorylase gene (GMP) plant expression vector p2301-GMP-myc into lettuce, while the content of vitamin C determined by HPLC-ELSD of most transgenic lettuce was higher than normal plants, even up to about 2.5 times, which have shown that over-expression of the GMP gene was an effective method to improve the vitamin C content of lettuce (Wang et al., 2011). Meanwhile, *Lactuca sativa L.* was transformed with *Atpsy* and synthetic *folE* gene, and the expression of lutein, β-carotene and total folate content in transgenic lettuce were measured by Realtime-PCR, HPLC and *Lactobacillus casei* fermentation. Compared with the wild type, the content of lutein, β-carotene and folic acid increased much in transgenic lettuce, to our surprise, β-carotene content was three times and folic acid was 1.85 times (Fu et al., 2012). These studies laid the foundation for modifying its metabolic pathways by genetic engineering in the meantime obtaining the new lettuce varieties rich in vitamins, carotenoids and folic acid.

RESISTANCE RESEARCH OF TRANSGENIC LETTUCE

Although insect resistance, disease resistance and other excellent quality relative to economic traits were less studied, some breakthroughs have been made by researchers. For further work, resistance to Root Knot Nematodes and Aphids (Lipaphiserysimi) in transgenic lettuce (*Lactuca sativa*) made us better understand the mechanism of pest resistance (Zhang et al., 2010; Ahmed et al., 2007). Transgenic resistance to Mirafiori lettuce virus in lettuce carrying inverted repeats of the viral coat protein gene provided a new way to resist disease, and the MiLV-resistant lettuce could be used as a resistant cultivar or as a breeding source (Yoichi et al., 2009).The transgenic lettuce over-expression of *Arabidopsis A*BF3 gene showed higher tolerances than wild-type plants against drought as well as cold stress, which could help to develop stress tolerance with an eventual improvement in crop yield (Enkhchimeg et al., 2005).

THE PROSPECT OF APPLICATION

During the last 10 years, many advances in molecular biology and genetic engineering have been used to improve the agronomic and nutritional value of lettuce and introduce new attributes into existing cultivars. For genetic engineering efforts to be effective in delivering new cultivars, three interacting components are essential in tissue culture and genetic transformation. To start with, adventitious shoots induction of cotyledon was significantly influenced by genotype, so the research of explants types need to be strengthened; next, different types of hormone combination were mostly 6-BA and NAA,6-BA and IAA, so other hormone combinations should be explored to improve regeneration frequency; last but not the least, the existing transgenic lettuce was focused on the genes transformation and expression, whereas stable inheritance of the genes and genetic traits was less studied.

Most of them have been successfully integrated in developing transgenic lettuce, but various other issues need to be addressed and resolved. Even the environmental risks and health hazards associated with transgenic crops are still sources of concern to all countries in the world.

A trans-genetic lettuce and cucumber system containing rabbit defensin gene NP-1 were constructed and optimized by *Agrobacterium*-mediated method in my research. To examine the disease resistance of transgenic plant, a series of molecular detection and inhibition zone test were performed. The results revealed that the protein possessed good resistance to *E. coil* and *Staphylococcus aureus* (Song et al., 2013).

Although there are many problems that still need to be further explored, it can be said that the use of transgenic lettuce in production of pharmaceutical proteins and vaccines is a very effective one. Nowadays the transgenic soybeans have been approved by authorities, it is hoped that with proper assessment and field trials, many more transgenic crops will be applied in our everyday life in the future, including lettuce.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Influence of triadimefon on the growth and development of banana cultivars

Abdelnasser A. Galal[1]*, Ibrahiem A. Ibrahiem[2] and Jehan M. Salem[1]

[1]Botany Department, Faculty of Science, Sohag University, Egypt.
[2]Genetic Engineering Laboratory, Monofia University, Egypt.

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Triazole fungicide triadimefon (bayletone) is a broad systemic fungicide used in agriculture as screening agent. Triadimefon interferes with plant sterol biosynthesis leading to a changeable sterol profile, consequently morphological and cytological abnormalities. Its effect on banana cultivars was studied using shoot-tip cultures placed on Murashige and Skoog solid medium supplemented with 5 mg/L of 6-benzylaminopurine (BAP). The growth and proliferation of triadimefon treated shoot-tip cultures of the three-dessert banana cultivars (Hindi, Basrai and Williams) were affected compared to the control. The optimum culture conditions for root formation were obtained in the case of sub-culturing. The excised shoot cultures into Murashige and Skoog solid medium were supplemented with 1 mg/L indole-3-butyric acid (IBA). The efficiency of root system formation decreased as fungicide concentration increased. Many variations were observed among chlorophyll, carotenoids and protein contents of triadimifon (50 mg/L) treated cultures and untreated ones. High decrease was observed among the usual sterol content of triadimifon (50 mg/L) treated shoot buds compared to the control.

Key words: Banana, cultivars, fungicides, proliferation, shoot-tips, sterol biosynthesis, triadimefon, triazoles.

INTRODUCTION

Triazole fungicides could have side effect on the host plant, in some cases undesirable phytotoxic effects can occur which may limit or affect the growth and development (Gopi et al., 2008; Asami et al., 2003). Triazole compounds inhibit the 14-alpha-demethylation reaction in sterols biosynthesis by interacting with the cytochrome-P-450 monooxygenase of the 14-alpha-demethylase complex (Rahier and Taton, 1997), thus cause an accumulation of 14 -alpha-methyl sterols that cannot pack satisfactory with the fatty acylchains of the phospholipids of cell membrane (Piironen et al., 2000; Khalil et al., 1990), the formation of the latter is disrupted and plant growth is adversely affected (Kaspers, 2009; Asami et al., 2003). Plant growth retarding effect of triazole fungicide triadimefon associate with the accumulation of sterol precursors, delaying of seedling emergence and reducing of plant height, length of coleoptiles, primary leaves and roots (Abdul Jaleel et al., 2008;

*Corresponding author. E-mail: nasergalal@gmail.com.

Abbreviations: BAP, 6-Benzylaminopurine; **DMIs,** sterol demethylation inhibitors; **EtOH,** ethanol; **GLC,** gas-liquid chromatography; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog.

Table 1. Effect of different concentrations of triadimefon (bayleton) on the viability of shoot-tip explants of banana cultivars, Hindi, Basrai and Williams cultured on MS solid medium supplemented with 5 mg/L BAP for four weeks.

Concentration of triadimefon (mg/L)	Number of shoot-tip explants	% of the living shoot-tips mean ±SD		
		Hindi	Basrai	Williams
Control I	25	100 ± 0.0	100 ± 0.0	100 ± 0.0
Control II	25	100 ± 0.0	100 ± 0.0	100 ± 0.0
30	25	80 ± 0.40	72 ± 0.45	68 ±0.47
40	25	68 ±0.47	60 ± 0.50	32 ± 0.48
50	25	20 ± 0.40	12 ± 0.33	12 ± 0.33
60	25	04 ± 0.20	0.0 ± 0.0	0.0 ± 0.0
70	25	00±0.0	0.0 ± 0.0	0.0 ± 0.0

Control I = Shoot-tip explants cultured on MS solid medium + 5 mg/L BAP. Control II = Shoot-tip explants cultured on MS solid medium + 5 mg/L BAP + 1 ml/L EtOH.

Kishoreukmar et al., 2007). Triazoles fuingicides increases the ratio of Chl a to b in the treated plants (Gopi et al., 2008), affect the protein contents and the rate of photosynthetic rate of treated plant (Gomathinayagam et al., 2008; Lu et al., 2000). Triazoles fuingicides affect mitosis by a direct rather than indirect action on the build up or on the function of the mitotic apparatus (Al Mansouri and Kurup, 2009; Wetzstein et al., 2002). Based on this, the present study attempt to study the *in vitro* effect of triazole fungicide triadimefon on the growth and development of some banana cultivars and shed some light on its phytotoxic effect.

MATERIALS AND METHODS

Chemicals

Triazole fungicides, triadimefon (bayleton) from Bayer AG, Lever kuse, Germany, were kindly provided by Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. Triadimefon was solubilized in EtOH (Ethanol), and then it was added to the medium after autoclaving.

Plant material preparation

To determine the effect of triazole fungicide, triadimefon on the viability of shoot -tip explants of banana cultivars, Hindi, Basrai and Williams, shoot-tip explants (about 0.5 cm length) were cultured on MS solid medium (Murashige and Skoog, 1962) supplemented with 5 mg L^{-1} BAP and different concentrations (30, 40, 50, 60 and 70 mg/L separately) of triadimefon for four weeks. All cultures were incubated at the standard culture conditions of temperature (25 ± 2°C) and light regime (16 h/day) for 4 weeks. Absence of further growth was an indicator of lethality. To determine the effect of fungicide triadimfon on the growth and proliferation, rate of the excised lateral buds were cultured on the same solid medium (MS) supplemented with 5 mg/L BAP and at different concentrations (30, 40, 50 mg/L separately) of triadimefon. To determine the effect of fungicide triadimfon on the root formation shoots approximately 3 to 5 cm long were cultured on MS solid medium supplemented with 1 mg/L IBA and at different concentrations (l0, 20, 30, 40 and 50 mg/L separately) of triazole fungicide, triadimefon, then were incubated in the standard culture conditions as mentioned before. For all treatments two controls were used, the first one (control I) lacked the selective agent and the second one (Control II) lacked the selective agent but supplied with the dissolving agent as shown in the tables.

Chemical analysis of contents

Photosynthetic pigments (chlorophyll a chlorophyll b and carotenoids) were determined using spectrophotometric method as described by Metzner et al. (1965), the protein contents were determined colorimetrically according to the study of Lowery et al. (1951) and 4-demethyl sterols were determined according to AOAC (1984). Data were compared with the control ones.

Statistical analysis of data

Data were statistically analyzed by counting the means and standard deviation using the statistical package program for the social sciences (SPSS).

RESULTS

Banana cultivar shoot-tip explants of Williams were more sensitive to the fungicidal toxicity of triadimefon compared to the others (Table 1). Most of the shoot-tip explants failed to grow at the concentration of 60 mg/L triazole fungicide triadimefon, while two explants of Hindi and one of Basrai observed were growing at this concentration, but these shoot-tips seemed brown and weak in their phenotype. Results of the effect of different concentrations of triadimefon (Table 2), on the growth and proliferation rate of shoot-tip cultures of banana cultivars, showed that the number of shoots in all banana cultivars (Hindi, Basrai and Williams) decreased as well as triadimefon concentration increased, while the lengths of shoots decreased. The number of leaves per shoot showed a lower variation with the increase of triadimefon concentrations. The leaf lengths of hindi, basrai and Williams cultivars showed a lower decrease with the increase of triadimefon concentration compared to the control (Table 2). The results of the effect of different concentrations of triazole fungicide triadimefon on the

Table 2. Effect of different concentrations of triadimefon on the growth and proliferation rate of shoot-bud explants of banana cultivars, Hindi, Basrai and Williams cultured on MS solid medium supplemented with 50 mg/l BAP for four weeks. Values are mean of 25 replicates per treatment in five jars ± SD.

Cultivar	Treatment (mg/L)	Number of shoots/explant (cm)	Length of shoots (cm)	Number of leaves /shoot	Length of leaves (cm)
	Control I	5 ± 0.3	1.01 ± 0.07	4.00 ± 0.40	0.80 ± 0.07
	Control II	4 ± 0.4	1.12 ± 0.08	2.00 ± 0.28	0.90 ± 0.07
Hindi	30	3 ±0.5	0.98 ± 0.04	2.00 ± 0.47	0.73 ± 0.03
	40	2 ± 0.4	0.93 ± 0.02	2.00 ± 0.51	0.70 ± 0.03
	50	1 ± 0.3	0.91 ± 0.06	1.00 ± 0.48	0.62 ± 0.04
	Control I	4 ± 0.4	1.00 ± 0.04	3.00 ± 0.33	0.72 ± 0.03
	Control II	3 ± 0.4	1.34 ± 0.05	2.00 ± 0.20	1.10 ± 0.04
Basrai	30	3 ± 0.6	1.03 ± 0.08	2.00 ± 0.51	0.76 ± 0.05
	40	3 ± 0.5	0.90 ± 0.04	2.00 ± 0.36	0.70 ± 0.03
	50	1 ± 0.4	0.80 ± 0.04	1.00 ± 0.38	0.60 ± 0.06
	Control I	5 ± 0.5	1.14 ± 0.05	3.00 ± 0.37	0.90 ± 0.02
	Control II	4 ± 0.5	1.10 ± 0.05	2.00 ± 0.43	0.80 ± 0.05
Williams	30	3 ± 0.3	1.10 ± 0.03	2.00 ± 0.29	0.80 ± 0.06
	40	3 ± 0.5	0.90 ± 0.03	2.00 ± 0.50	0.70 ± 0.04
	50	1 ± 0.0	0.80 ± 0.05	1.00 ± 0.48	0.60 ± 0.03

Control I = MS solid medium + 5 mg**/l** BAP. Control II = MS solid medium + 5 mg**/l** BAP + 1 ml**/l** EtOH..

excised shoots rooting of the three-dessert banana cultivars showed decrease in the number of roots per shoot, also decrease in the average lengths of roots with the increase of fungicide concentration (Table 3). The content of various pigment fractions (ChI a, ChI b and carotenoids) of Hindi and Williams, triadimefon treated cultivars showed a little differences compared to the control. The ratio of ChI a / ChI b and the total content showed a lower decrease in the case of Hindi cultivar, and lower increase in the case of Williams cultivar. An increase in various pigment fractions (ChI a, ChI b and carotenoids) contents and the total content of Basrai cultivar was observed compared to control I, but showed a lower decrease in the ratio of ChI a / ChI b (Table 4). Littlie variation in the three cultivar pigment fragments contents in the case of control II were recorded in comparison with each others. The total pigment content of control II showed increase in the case of Basrai cultivar compared to other cultivars and control I. The same result was obtained with control II of Williams cultivar compared to control I of the same cultivar. In the case of Hindi, it was showed that ChI a, carotenoids and the total content of control II showed lower decrease compared to those of other ones and those of control I (Table 4). However, there is no relationship between the effect of the fungicide and its dissolving agent on the pigmentation when the effect of fungicides on the pigments is well obvious phenotypically and analytically compared with those of control I or control II.

GLC analysis for the unsaponifiable matter of banana cultivars developed shoots under the effect of triadimefon (50 mg/L), revealed that the relative percentage of Δ^5 sterols (sitosterol, stigmasterol and campesterol) decreased compared to the control (Table 5), since dramatic quantitative reduction in the total percentage of sterols was observed compared with the control.

The results of protein chemical analysis showed that the total protein content of triadimefon (50 mg/L) treated shoots of Hindi showed little decrease compared to the control (Figure 1a). The soluble protein content of Hindi treated shoots was approximately equal to the control II content. The insoluble proteins content of triadimefon-treated shoots of Hindi cultivar showed a lower decrease compared to those of control I and control II respectively. The total protein content of Hindi cultivar triadimefon (50 mg/L) treated shoots showed a relatively high decreasing compared to control ones. While, in the case of Basrai cultivar showed a highly increase compared to the control (Figure 1b). The total protein content showed a highly increase compared to control I and control II, the same results were obtained in the case of Williams. In the case of Basrai cultivar, the insoluble proteins content of the triadimefon-treated shoots showed an increase in comparison to those of the control. The results show that the total proteins content of Williams cultivar (Figure 1c) treated shoots was higher than those of the control. High increase in the insoluble proteins content of triadimefon treated shoots of Williams cultivar compared to those of

Table 3. Effect of different concentration of tiazole fungicide triadimefon on the rooting of the excised shoots of the three banana cultivars, Hindi, Basrai and Williams cultured on Ms solid medium supplemented with 1mg**/l** IBA for three weeks.

Cultivar	Treatment (mg/L)	No of excited shoots	No of rooted shoots	No of roots /shoot	Length of roots
Hindi line	Control I	25	100	6 ± 0.83	8.8 ± 1.60
	Control II	25	100	6 ± 0.57	3.0 ± 0.35
	10	25	93	4 ± 0.53	0.5 ± 0.04
	20	25	73	2 ± 0.51	0.4 ± 0.09
	30	25	60	2 ± 0.50	0.3 ± 0.04
	40	25	20	1 ± 0.50	0.2 ± 0.00
	50	25	00	0 ± 0.00	0.0 ± 0.00
Basrai line	Control	25	100	5 ± 0.57	5.0 ± 1.06
	Control II	25	100	8 ± 0.50	3.0 ± 0.70
	10	25	93	4 ± 0.46	0.5 ± 0.04
	20	25	40	2 ± 0.57	0.5 ± 0.05
	30	25	26	1 ± 0.00	0.3 ± 0.05
	40	25	0	0 ± 0.00	0.0 ± 0.00
	50	25	0	0 ± 0.00	0.0 ± 0.00
Williams line	Control I	25	100	6 ± 0.95	2.5 ± 0.25
	Control II	25	100	5 ± 1.51	4.5 ± 0.70
	10	25	100	4 ± 0.57	0.6 ± 0.20
	20	25	60	3 ± 0.00	0.5 ± 0.05
	30	25	20	1 ± 0.00	0.5 ± 0.00
	40	25	08	1 ± 0.00	0.2 ± 0.00
	50	25	0	0 ± 0.00	0.0 ± 0.00

Control I = MS solid medium + 5 mg**/l** BAP. Control II = MS solid medium + 5 mg**/l** BAP + 1 ml**/l** EtOH..

Table 4. Photosynthetic pigments content of banana cultivars, Hindi, Basrai and Williams cultured on MS solid medium supplemented with 5 mg/l BAP and 50 mg/l triadimefon for four weeks.

Cultivar	Treatment	Pigment content (mg/g fresh weight)				
		Chl a	Chl b	Carotenoids	a/b	Total
Hindi line	Control I	0.27 ± 0.03	0.10 ± 0.00	0.09 ± 0.01	2.70	0.46
	Control II	0.14 ± 0.04	0.11 ± 0.05	0.08 ± 0.02	1.27	0.33
	50 mg/l	0.22 ± 0.11	0.19 ± 0.00	0.07 ± 0.01	2.44	0.38
Basrai line	Control I	028 ±0.01	0.15 ± 0.01	0.06 ± 0.01	1.87	0.49
	Control II	0.34 ± 0.02	0.20 ± 0.01	0.09 ± 0.00	1.70	063
	50 mg/l	0.35 ± 0.04	0.20 ± 0.04	0.10 ± 0.01	1.75	0.65
Williams line	Control I	020±0.01	0.15 ±0.02	0.06 ± 0.00	1.33	0.4l
	Control II	0.32 ± 0.110	0.20 ± 0.00	0.09 ± 0.030	1.60	0.61
	50 mg/l	0.23 ± 0.030	0.16 ± 0.0007	0.07 ± 0.007	1.44	0.46

Control I = Shoot-tip explants cultured on MS solid medium +5 mg/l BAP. Control II = Shoot-tip explants cultured on MS solid medium +5 mg/l BAP + 1 ml/l EtOH. Values are means of three replicates ± SD.

the control and other cultivars contents was observed. The soluble protein contents of untreated shoots of the three cultivars (Hindi, Basrai and Williams) were approximately equal. While the insoluble proteins content of Basrai and Williams cultivars showed a lower decrease compared with those of Hindi cultivar.

Table 5. Effect of triazole fungicide triadimefon (50 mg/l) on the 4-dementhyl sterols (sitosterol, stigmasterol and campesterol) content of the three banana cultivars shoots cultured on MS solid medium supplemented with 5 mg/l BAP for four weeks.

Treatment	Hindi line				Basrai line				Willimas line			
	Sitosterol	Stigmasterol	Campesterol	Total	Sitosterol	Stigmasterol	Campesterol	Total	Sitosterol	Stigmasterol	Campesterol	Total
Control	2.1	20.6	5.1	27.8	6.7	2.5	3.9	13.1	11.9	5.7	12.5	30.1
50 mg/L	1.1	16.9	3.4	21.4	0.3	2.1	0.3	2.7	6.7	2.8	2.4	11.9

- % was calculated as relative to the total percentage of unsaponifiable matter. - Control = MS basal medium + 5 mg/l BAP.

DISCUSSION

Plant cell is a dynamic living system response to systemic fungicides by complex series of biochemical changes. Systemic fungicides enter the plant and encounter a variety of physiological and biochemical changes. The results of our work showed that the viability of shoot-tip explants of the three banana cultivars, Hindi, Basrai and Williams decreased as well as the concentration of triadimefon increased. These results are in agreement with those mentioned by Gopi et al. (2008) and Lu and Guo (2000) who reported that triazole fungicides affected many plant growth properties. The obtained results of this study indicated that the phytotoxic effect of triadimefon includes reduce in surface area of leaves and growth retardation of shoots and roots. This conclusion is in agreement with those reported by Abdul Jaleel et al. (2008) and Kishorekumar et al. (2007), who reported that the phytotoxic effect of triadimefon was parallel to the inhibition of plant sterol biosynthesis rather than gibberellins biosynthesis. Many explanations have been given by many investigators regarding the decrease of growth and proliferation rates (Kaspers, 2009; Khalil et al., 1990). The suppression of growth could be attributed to the inhibition of the enzyme (cytochrome P-450-dependent obtusifoliol-14-demethylase) responsible for the removal of the C-14 methyl group, led to the accumulation of 14-alpha-methyl sterols at the expense of Δ5-sterols (sitosterol, stigmasterol and campesterol) this observation is in agreement with the study of Lu et al. (2000) . The plant growth retardant effect of triadimefon may be associated with an inhibition of the biosynthetic pathway of of campesterol (Asami et al., 2003), whereas brassinosteroids has been shown via two pathways from campesterol. Brassinosteroids are plant sterols that cause cell elongation, cell expansion, enhances gravitropism, retard abscission and promote xylem differentiation (Asami et al., 2003; Hartmann, 1998). Δ5-Sterols play an important metabolic role in the cell proliferation process (Piironen et al., 2000) who reported that stigmasterol might be specifically required for cell proliferation. Lower concentrations of stigmasterol were unable to restore growth of celery cells treated with an inhibitor of the obtusifoliol 14-demethylase, but a combination of low concentration of stigmasterol together with a high concentration of cholesterol was effective as a relatively high concentration of stigmasterol alone (Hartmann, 1998; Kisorekumar et al., 2007) pointed out that the triazole fungicides inhibit the 14-demethylation reaction in plant sterols biosynthesis by interacting with the cytochrome -P-450- monooxygenase of the 14-alpha- methyl sterols that cannot pack satisfactory with the fatty acyl chains of the phospholipids of the plant membrane. The formation of the latter is disrupted and the plant growth is adversely affected. Wetzstein et al. (2002) repotted that triazoles fungicides affect mitosis by a direct rather than indirect action on the build up or on the function of the mitotic apparatus so spindle damage could be caused by interference with microtubule polymerization or with the replication of the spindle organizing center or increase the frequency of abnormalities such as chromosome clumping at metaphase and anaphase. Al Mansouti and Kurup (2009) and Kaspers (2009) reported that triazole fungicides might be interfere with the membrane vesicle which in close association with microtubules. The effect of DMIs on the biosynthesis of chloroplast pigments remained unclear. The intense greening of leaves of the treated shoots in the sub-lethal concentration (50 mg L^{-1}) of triadimefon may be attributed to the increase in ChI concentration per unit area of leaf. This observation is in agreement with those of Gomathinayagam et al. (2008) and Kisohrekumar et al. (2007), whereas the greening effect might be associated with the growth retarding activity of the fungicides. Pigments probably condensed into a smaller leaf area of the treated shoots, which appeared darker green than control ones. These observations correspond to those of Abdul Jaleel et al. (2008), Gopi et al. (2008) and Khalil et al. (1990) who reported that triazole fungicides were ineffective in changing the ChI content (per unit fresh weight). It appeared

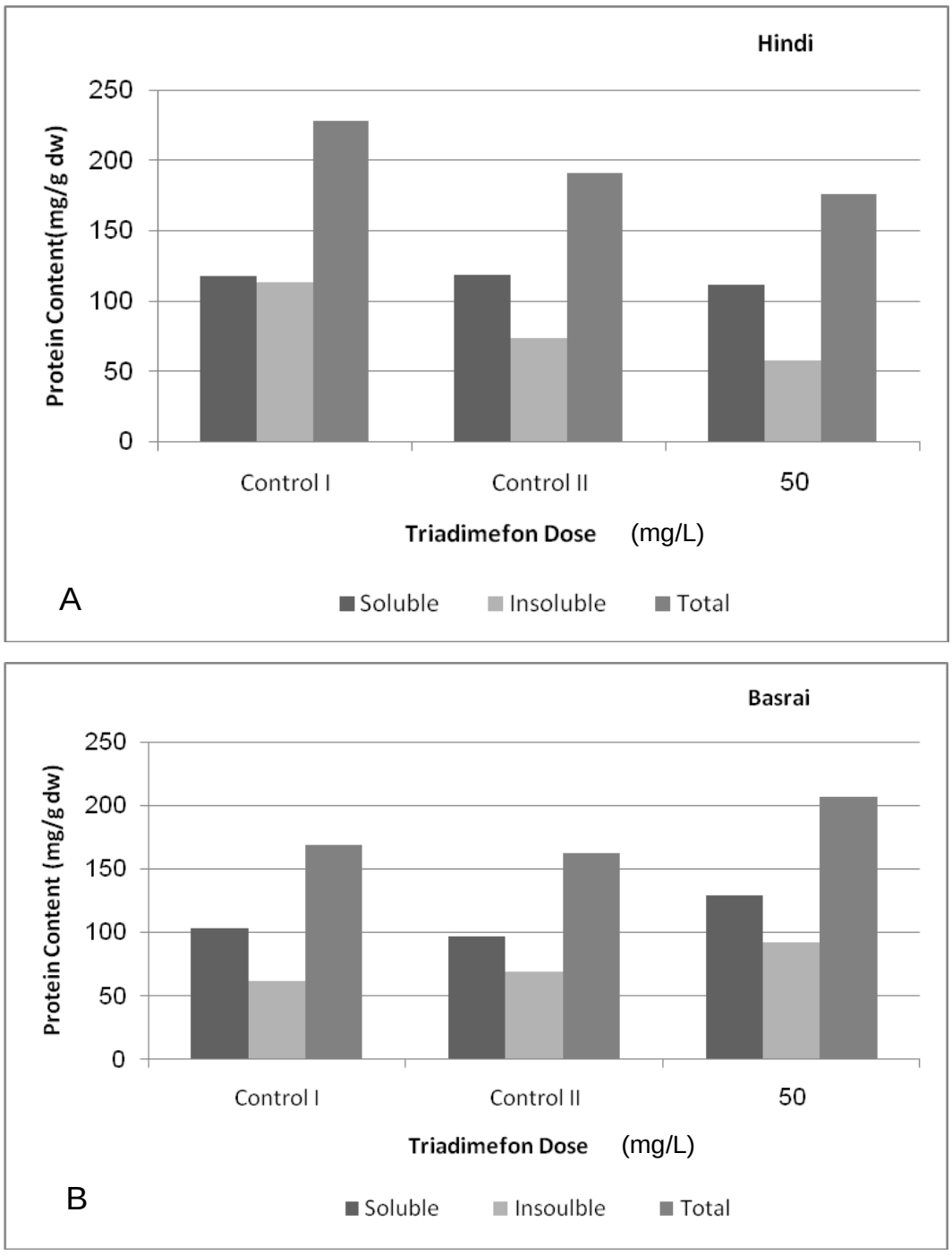

Figure 1. Showing the effect of triadimefon on soluble, insoluble and total protein contents (mg/g dw) of **(A)** Hindi (B) Basrai (C) Williams shoots cultured on Ms solid medium supplemented with 5 mg/L BAP + 50 mg/L triadimefon for four weeks. Control I = Shoot-tip explants cultured on MS solid medium + 5 mg/L BAP. Control II = Shoot-tip explants cultured on MS solid medium + 5 mg/L BAP + 1 ml/L EtOH. Values are means of three replicates.

that changes observed in the ChI and carotenoids content of the treated shoots with triadimefon were related to the growth retardation of the fungicide, but the ratio of ChI a / ChI b was not influenced by it. This showing that the triazole fungicide, triadimefon had no immediate effect on the carotenoids hydroxylation systems in the leaf; this result are in agreement with those obtained by Kishorekumar et al. (2007) and Abdul

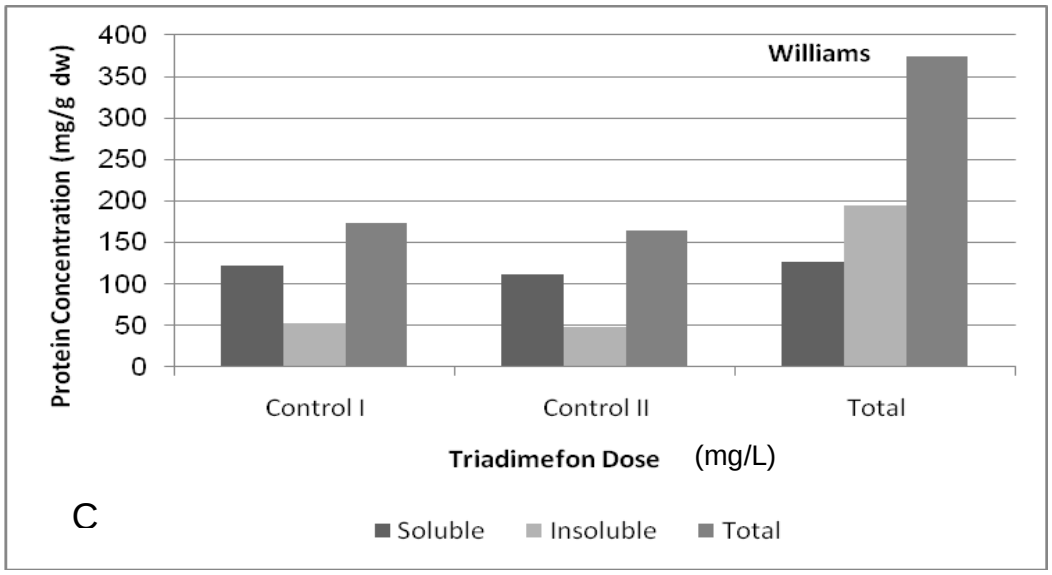

Figure 1. Continued.

Jaleel et al. (2008). There is no evidence that triazole fungicides inhibit all cytochrome P-450 mixed functions oxygenases like those of sterol or GA biosynthesis. Protein synthesis is essential for normal cell proliferation and differentiation. Increasing or decreasing of protein contents could be attributed to the differences in the physiological and morphological characters of each cultivar (Kaspers, 2009). It is well known that fungicides reflect a type of particular stress conditions exhibit alteration of gene expression inducing a change in the plant metabolism resulting in an alteration in the protein synthesis which may vary according to the phenotype of plant (Hy et al., 2002; Schrick et al., 2000). In conclusion this study revealed that the systemic triazole fungicide triadimefon might have an inhibitory effect on the morphology and physiology of higher plants, which appeared *in vitro* on the growth and development of the three desert banana cultivars, Hindi, Basrai and Williams and its inhibitory effect might be attributed to its phytoxic effect or its accumulation in plant tissues.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Use of plumules cryopreservation to save coconut germplasm in areas infected by lethal yellowing

Oulo Alla-N'Nan[1,2]*, Sery Gonédelé Bi[1], Kouakou Tiécoura[1], Jean-Louis Konan Konan[3] and Bernard Malaurie[2]

[1]Université Félix Houphouet Boigny, 22 BP 582 Abidjan 22, UFR Biosciences, laboratoire de génétique, Côte d'Ivoire.
[2]IRD, UMR DIADE, 34394 Montpellier Cedex 5, France.
[3]Centre National de Recherche Agronomique (CNRA), Programme cocotier, Station de recherche Marc Delorme, 13 BP 989 Abidjan 13, Côte d'Ivoire.

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Plumules excised from zygotic embryos through the largest representative diversity of four of the five different areas of coconut cash and food crops were used in a cryopreservation process using encapsulation-dehydration technique. Five accessions of coconut trees were used [Panama Tall (PNT/GPA), Brazilian Green Tall (BGD/NVB), Cameroon Red Dwarf (CRD/NRC), Vanuatu Tall (VTT/VNT/GVT), and Tagnanan Tall (TAGT/GTN)] in addition to the accession model [Malayan Yellow Dwarf (MYD)] from which an optimal protocol was obtained. A great variability of response was observed depending on accessions with survival and growth recovery rates varying from 6 to 66% and 0 to 24% after 2 and 7 months of culture, respectively.

Key words: Coconut, accessions, germplasm, plumules, cryopreservation, encapsulation-dehydration.

INTRODUCTION

Coconut germplasm is subjected to an increasing genetic erosion based on it particular germplasm conservation. Its mode of conservation based on field collections, because of the characteristics of the seed (no dormancy and recalcitrant to storage), exposes collections to climatic adversity, pest and diseases. Among coconut diseases, lethal yellowing (LY) is actually the most dreadful (Dollet, 1999). It has devastated hundreds of thousand hectares throughout the world (Africa, Latin-America, and Caribbean). This disease is caused by the phytoplasma's presence in the phloem. The use of zygotic embryo for exchanges and conservation of germplasm can be tricky because their tissues contain differentiated vascular system in which the pathogen can be maintained (Harrison et al., 1995; Cordova et al., 2003). In this context, the use of plumule, composed of the apical dome with three or four leaf primordia excised from coconut zygotic embryo, was presented as an attractive approach to coconut cryopreservation as it has only provascular strands without differentiated phloem (N'Nan et al., 2008). International germplasm exchange amplification between countries belonging to Cogent

*Corresponding author. E-mail: nanoulo@yahoo.fr.

Table 1. List of coconut accessions used by culture area (French and English appellation).

"Tall" Accessions	Culture areas	"Dwarf" Accessions	Culture areas
Grand du Vanuatu (GVT) / Vanuatu Tall (VTT/VNT)	South Pacific, Vanuatu	Nain Rouge Cameroun (NRC) / Cameroon Red Dwarf (CRD)	Africa, Cameroon
Grand Panama (GPA) / Panama Tall (PNT)	Latin-America, Panama, Pacifique coast	Nain Vert Brésil (NVB) / Brazilian Green Dwarf (BGD)	Latin-America, Brazil
Grand Tagnanan (GTN) / Tagnanan Tall (TAGT)	South-East Asia, Philippines	Nain Jaune Malais (NJM) / Malayan Yellow Dwarf (MYD)	South-East Asia, Malaysia

(Coconut germplasm network) and laboratories from the South imply the use of disease-free planting material (Morel and Martin, 1952). The plumule is therefore expected to limit the risk of some disease transmission such as lethal yellowing (Frison et al., 1993; Malaurie, 2001; Hocher et al., 2004). The use of plumule as germplasm exchange material and its cryopreservation are an effective approach to preserve disease-free planting material, particularly when the material comes from infected areas

The recent works done by N'Nan et al. (2008) showed that cryopreservation of coconut plumules is possible after freezing in liquid nitrogen at -196°C. This article evaluates the plumule cryoconservation of «Talls» and «Dwarfs» coconut accessions. These accessions in addition to the Malayan Yellow Dwarf (MYD) are common in coconut culture areas.

MATERIALS AND METHODS

Plant material

Plumules tissues (caulinary meristem surrounded by three to four leaf primordia) were excised from mature zygotic embryos (10 to 12 months after pollination). The nuts in the form of endosperm cylinders or albumen cores were supplied by the Marc Delorme Research station of CNRA, Côte d'Ivoire. Six accessions were used (Table 1): three accessions "Dwarf" (Malayan Yellow Dwarf (MYD), Brazil Green Dwarf (BGD), and Cameroon Red Dwarf (CRD)), and three accessions "Tall" (Panama Tall (PNT), Vanuatu Tall (VTT), and Tagnanan Tall (TAGT)).

Plant material extraction, disinfection and conditioning

The material originated from Côte d'Ivoire in the form of endosperm cylinders containing embryos. The extraction and disinfection of endosperm cylinders before dispatching was performed in Côte d'Ivoire as previously described by Assy Bah et al. (1987). For their conditioning before mail invoice, rinsed endosperm cylinders were transferred in cleaned, disinfected, plastic bags. They are then packaged in small plastic bags of 10 and sealed in a biger plastic bag up to 100. Each transportation mailing may concern up to 4 big plastic bags, put in a polystyrene box filled with several plastic bags containing frozen water. The material was kept as long as possible under refrigeration before leaving it to the mailing post service companies (preferably DHL). After receiving the endosperm cylinders, their disinfection anew, the extraction of the embryo contained in these endosperm cylinders and the excision of plumules from embryos were carried out following the protocol described by Malaurie et al. (2006) and N'Nan et al. (2008).

In vitro culture (medium and culture conditions)

The medium and the culture conditions applied to these accessions have been previously described by N'Nan et al. (2008). After different step of cryopreservation plumules were placed at 27°C in the dark, until the first 3 to 4 leaves emerged. Then, they were exposed to a daily photoperiod of 12 h with light intensity of 45µE m^2s^{-1}.

Cryopreservation (encapsulation, pre-atrement-dehydration and freezing)

For encapsulation, the plumules were suspended in standard medium solution containing 3% (v/v) Na-alginate and 0.15 M sucrose. The plumules-containing mixture was dispensed with a sterile pipette into 0.1 M calcium chloride (CaCl2) solution containing 0.15M sucrose at room temperature to form beads (about 3 to 4 mm in diameter), with each bead containing one plumule. Thereafter, the beads were pretreated for 2 to 3 days sequentially in standard medium (without Gelrite and activated charcoal) containing two sucrose concentrations (0.75 M and 1 M). Up to 20 beads for each accession were put in each 125 ml Erlenmeyer flask containing 30 ml medium and shaken on a rotary shaker set at 90 to 100 rpm, at room temperature. After pretreatment with sucrose, the beads were dried to remove excess pretreatment medium. They were placed to dehydrate for 8 h (1 M) or 16 h (0.75 M) on sterile filter paper over 40 g silica gel in 125 ml airtight boxes. Up to 20 beads were put in each airtight box. Following dehydration, half of the beads (ten) were transferred to standard medium. The other half were transferred into a 2 ml cryotube and immersed directly in liquid nitrogen for at least 2 h. Thawing was performed by immersing the cryotubes in a water bath at 40°C for 3 min. Each cryopreserved bead was then transferred to a test tube filled with standard medium. Three replicates of each treatment have been done.

Evaluation of survival and recovery

The effect of pre-treatment and freezing on the plumule are evaluated by determining the percentage of survival and the percentage of recovery. Plumules were considered alive when they increased in size from 1 mm to about 3 mm and more after 1 to 3 months. Recovery is considered normal if plumules growed and produced shoots and leaves after at least eight months. Dehydrated and unfrozen (pre-treated) material is noted –LN and the dehydrated and frozen material is noted +LN. Unfrozen material allowed seeing the effect of dehydration which is the most difficult step for recalcitrant seeds.

Table 2. Survival and recovery rate of plumules from different accessions in function of pretreatment and dehydration duration.

Pretreatment (d / M / h)		Accessions											
		MYD		BGD		CRD		PNT		VTT		TAGT	
		PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2	PT1	PT2
Survival (%) ***F=0.5809 ; P= 0.799***													
Freezing	LN -	60 ± 15.3	32.8 ± 3.6	21 ± 9	31.1 ± 8.9	84.5 ± 4.4	61.1 ± 5.5	60	75 ± 25	0	36.3 ± 23.8	18.3 ± 4	0
	LN +	50 ± 5.8	40 ± 5.8	5.6 ± 5.5	0	67.5 ± 7.5	48.8 ± 12.3	12.5 ± 12.5	50 ± 20	0	25 ± 5	5 ± 5	0
Recovery (%) ***F= 0.4858 ; P=0.7439***													
Freezing	LN -	30 ± 9.5	21.4 ± 4.6	0	0	52.8 ± 2.8	10.6 ± 0.6	35 ± 5	30 ± 20	0	0	5.6 ± 5.5	0
	LN +	23.3 ± 6.5	20 ± 5.8	0	0	36.3 ± 26.3	10 ± 10	0	20 ± 10	0	0	5 ± 5	0

Malayan Yellow Dwarf (MYD), Brazilian Green Tall, Cameroon Red Dwarf (CRD), Panama Tall, Vanuatu Tall, Tagnanan Tall. The given values correspond to the mean of obtained measures after 3 replicates. Survival and recovery rate are measured from 2 months and 5 months of culture, respectively. One ANOVA factorial was used for the analysis. When the test is significant, $P < 0.05$, as the test was not significant, none classification was done. *PT1*: 2 days with 1 M and 8 h dehydration; *PT2*: 3 days with 0.75 M and 16 h dehydration; *LN-* : unfrozen plumules; *LN+*: frozen plumules, d / M / h: days/concentration of sucrose/hours.

Statistical analyses

Treatments were arranged in a randomized complete block and each treatment was replicated three times. ANOVA factorial or one-way ANOVA were used to determine treatment effects. When significance was indicated ($P \leq 0.05$), the least significant difference was calculated at the level of 5% by Newman–Keuls test (Newman, 1939; Keuls, 1952).

RESULTS

The effects of two treatments (3 days with 0.75 M sucrose/ 16 h dehydration, and 2 days with 1 M sucrose/ 8 h dehydration) are indicated in Table 2. Although these results do not show significant differences, some observations can be pointed out.

The survival and growth recovery rates vary in function of accessions and treatment used. With Panama Tall (PNT), the plumules pre-treatment with 0.75 M sucrose followed by 16 h dehydration seems to allow more interesting results for dehydrated unfrozen (–LN) and dehydrated frozen (+LN) plumules with 75 and 50% survival rates, respectively. The growth recovery rate, as far as it is concerned, is of 30% for (–LN) and 20% for (+LN). More interesting results were obtained with Cameroon Red Dwarf (CRD), when pre-treatment is done with 1 M sucrose followed by 8 h dehydration. For this accession, survival rates reached 84.5 and 67.5% for (–LN) and (+LN), respectively; this was also observed for the growth recovery with 52.8 and 36.3 % for (–LN) and (+LN), respectively. With this accession, higher survival and growth recovery rates have been obtained than previously observed with the MYD accession. The 3 other accessions react to the 2 treatments with survival and growth recovery rates, lowest than those observed with MYD. No survival and growth recovery were obtained with VTT plumules when treatment is done with 1 M sucrose. When pre-treatment is done with 0.75 M sucrose, the plumule survival rate vary from 25 to 35% for (–LN) and (+LN) plumules, respectively. On the other hand, no growth recovery was observed. Similar results were obtained with Brazil Red Dwarf (BRD). With regards to the survival response of this accession, it still remains low overall for frozen plumules (0% for 16 h and 5.56% for 8 h). For Tagnanan Tall (TAGT), no survival and growth recovery was observed when 0.75 M sucrose concentration is used for pretreatment followed by 16 h dehydration. Very low survival and growth recovery rates of about 5% were obtained when 1 M sucrose is used.

Highly significant differences ($P < 0.001$) were obtained between accessions when factor accession is only considered (Table 3). On the level of survival rate, the CRD is the accession which presents the best rate, followed by the PNT. In other group, the BGD, TAGT, and VTT accessions present lower survival rates ($P = P < 0.001$). A quite equivalent distribution is observed for the growth recovery rate, except for CRD and PNT which have the highest growth recovery rates.

Table 3. survival and recovery rate of plumules in function of accessions.

Accessions	Survival (%)	Recovery (%)
MYD	40.7 ± 7.6 [b]	23.7 ± 6.6 [a]
PNT	49.4 ± 10.8 [b]	21.3 ± 6.6 [a]
BGD	14.4 ± 5.3 [c]	0 [b]
CRD	65.5 ± 5.6 [a]	27.4 ± 8.6 [a]
VVT	15.3 ± 7.5 [c]	0 [b]
TAGT	5.8 ± 3 [c]	2.6 ± 1.7 [b]
	F = 23.9407	*F = 9.91395*
	P = 0.000000	*P = 0.000137*

The given values correspond to the mean of all the treatments done for a given accession. It combines the entire mean obtained at all the dehydration durations, the mean obtained with all the frozen and unfrozen plumules, knowing that all treatment are the mean obtained over 3 replicates. ANOVA factorial was used for the analysis. Values, in the same column, when followed by the same letter are not significantly different according to a Newman and Keuls test at *P<0.05* Newman (1939); Keuls (1952). Legend : MYD : Malayan Yellow Dwarf ; BGD : Brazilian Green Dwarf ; CRD : Cameroon Red Dwarf ; PNT : Panama Tall ; VTT : Vanuatu Tall ; TAGT : Tagnanan Tall.

DISCUSSION

Two treatments have been proposed to evaluate the plumule cryoconservation of coconut “Talls” and “Dwarfs” accessions. The first work on coconut plumule cryopreservation was reported in 2001 (Hornung et al., 2001). The authors obtained embryogenic callus after cryopreservation and post culture in media containing growth regulators such as 2,4 D. Our present work indicates that the application of these treatments to five accessions (BGD, TAGT VTT, CRD, PNT) give underwhelming results. While these treatments seem to be inappropriate to BGD, TAGT and VTT, they give good result with CRD and PNT where similar or higher growth recovery and survival rates compare to these obtained with MYD are observed. These results can be explained by the heterogeneity of the material used (seeds from open pollination), and the conditions of cryopreservation in general. Indeed cryopreservation requires a relatively large number of materials and a lot of repetitions that cannot be performed for materials such as coconut (slow development, difficulty in plumules excision). As for recalcitrant material in general, the dehydration of the plumule tissues is the most difficult step of cryopreservation (Chandel et al., 1995; N’nan et al., 2008; 2012). Freezing cannot be done without dehydration and dehydration of recalcitrant material without any protection (pre-treatment) leads to a loss of viability (N’nan et al. 2008). Pre-treatment is essential for allowing the material to withstand water loss (Ref). In this study, the use of two sucrose concentrations at different times (pre-treatment) help the plumules of some accessions to support dehydration and freezing.

A high concentration of sugar which causes rapid dehydration by osmotic dehydration must be performed for a short time inversely. Indeed further dehydration cause the loss of bound water and damage of the material. Sugar used played a cryoprotectant role to offset the loss of water (free water) essential for freezing. In this study, no survival was obtained with slow freezing. This confirms the work of Berjak et al. (2000); Dussert et al. (2001) who indicated that for recalcitrant material, rapid freezing cause water vitrification by contrast to slow freezing which resulted in the formation of ice crystals that damage cells. The lack of survival without recovery for the plumules of some accessions suggested irreversible damage caused by dehydration as shown by several authors through estructurales studies (Pammenter et al., 1999; N’nan et al., 2008). According to Wilkinson et al. (2003) regenerating a plant came from several areas which are located between the dome and leaf primordia. Depending on the damage to these areas after dehydration and freezing, and the origin of regeneration, the material will undergo different damages in its development.

The accessions used in this study have been demonstrated to differ genetically (Perera et al., 2000; Dasanayaka et al., 2009). Such differences may contribute to the variable recovery properties of distinct accessions. ‘Dwarf’ accessions, notably MYD and CRD, are more tolerant to cryopreservation than ‘Tall’ accessions, principally West African Tall (WAT) Sri Lnaka Tall (SLT). Tall accessions are generally considered more recalcitrant to *in vitro* culture and cryopreservation notably WAT and SLT (Assy Bah and Engelmann, 1992, N’Nan et al., 2012). Malaurie et al. (2006) with SLT obtained 9% of recovery after addition of abscisic acid (ABA), showing the recalcitrance of this accession to cryopreservation. In contrast Bandupriya et al. (2007; 2010) obtained a better recovery on the same variety testing the effect of storage and the effect of the concentration of ABA on cryopreservation (30%). While

the genotype of some accessions can tolerate the condition of cryopreservation other genotype does not. Heterogeneity of material is a limiting factor for the cryopreservation of seeds species (Pammetier and Berjak, 1999). The same observation was made with coconut zygotic embryo. Despite the availability of a protocol that gives interesting results with some accessions, others accessions seem to have lower results (N'nan et al., 2012). Cryopreservation of the plumules is a recent study, the improvement of the protocol for its adaptation to a large number of accessions is essential. The plumule is an ideal material for the conservation of germplasm through cryo-preservation especially for areas affected by lethal yellowing. Although improvements are still to be done, the present treatment applied to plumules excised from embryos provided from recalcitrant seeds is encouraging and allows starting a cryobank. This could help to preserve a large portion of germplasm in affected areas.

Conclusion

This study indicates that accessions react differentially to cryopreservation process. For some accessions a complete revision of the protocol is needed, while for others, only some improvements are needed. Our results show that cryopreservation of plumules is possible. Works should be continued to define all the difficulties and to know how to resolve them.

However, the use of complete zygotic embryo is still essential in the regions that are not infected by lethal yellowing disease. As long as embryos will be suspected for the transmission of lethal yellowing disease, plumules with their caulinary meristems will still constitute an ideal plant material, free of virus, ideal for phythosanitary exchange and cryopreservation.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from sea water

N. S. Nisha* and J. Divakaran

Department of Microbiology, Faculty of Science, Annamalai University, Annamalainagar- 608002, Chidambaram, Cuddalore District, Tamilnadu, India.

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The protease producing bacterial strain *Bacillus subtilis* was isolated from sea water and identified by 16S rRNA sequencing. The strain named as *B. subtilis* NS. Optimization of the strain revealed that the most suitable nitrogen source to enhance protease production was beef extract. Among various carbon sources tested, maximum production of protease was registered in medium with added glucose. The effect of metals ions indicated that maximum protease production was observed in medium supplemented with magnesium chloride (MgCl). Investigating the effect of sodium chloride (NaCl) concentration on protease production revealed that 7% yielded higher protease production. The most suitable pH and temperature for maximum protease production revealed that pH 9 and a temperature of 40°C gave optimal protease production.

Key words: Alkaline protease, *Bacillus subtilis*, media optimization, marine bacteria.

INTRODUCTION

Proteolytic enzymes are degradative enzymes which catalyse the cleavage of peptide bonds in other proteins. Alkaline protease, which works optimally in alkaline pH, constitutes 60 to 65% of the global industrial enzyme market (Amoozegara et al*.,* 2004). Proteases are the class of enzymes which occupy key position with respect to their applications in both physiological and commercial fields (Godfrey et al*.,* 1996). Protease derived from microorganisms such as bacteria, fungi and yeast has found wide spread applications in many fields. Among various proteases, bacterial proteases are most significant, compared with animal and fungal proteases (Fujiwara et al*.,* 1991). Alkaline protease of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, silver recovery, detergent, waste water treatment and reso-lution of amino acid mixtures (Rao et al*.,* 1998). Currently a large proportion of a commercially available alkaline proteases are *Bacillus* strains (Yang et al., 2000) although several fungal sources are being increasingly employed (Banerjee, 1999). Among these, *Bacillus subtilis* is the most important group of bacteria that are involved in the enzyme industries and also *B. subtilis*

*Corresponding author. E-mail: nsnishanim@gmail.com.

produce a variety of extracellular and intracellular protease (JahirAlam Khan et al., 2011). In view, the present study was undertaken to optimize culture conditions for alkaline protease production by marine water isolate *B. subtilis* NS.

MATERIALS AND METHODS

Screening and isolation of proteolytic bacteria

The protease producing bacterial strain was isolated from sea water of Cuddalore coast, Tamilnadu, India, using Zobell marine agar medium and plates were incubated at 37°C for 5 days (pH 9.0). It was identified by morphological, biochemical identification schemes and confirmed by 16S rRNA gene sequencing. In brief, DNA was isolated by phenol chloroform method (Marmur, 1961). The primer sequences were selected from the conserved regions as previously reported for the bacterial 16S rRNA gene (Saitou et al., 1987). Sequencing was done using forward primer (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (5′-GGGCGGTGTGTACAAGGC-3′). PCR were performed with following conditions: 35 cycles consisting of 95°C for 1 min and 72°C for 5 min, followed by final extension of 5 min at 72°C. The 16S rRNA gene sequences were obtained by an automated DNA Sequencer (Megabace, GE) and homology of the isolated gene with sequences in the Gene Bank database was analyzed.

Enzyme production medium

Production medium contained glucose 0.5 g (W/%), peptone 1 g, $FeSO_4$ 0.1 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.5 g and NaCl 3 g. 10 ml of medium was taken in a 100 ml conical flask. The flasks were sterilized in autoclave at 121°C for 15 min and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 48 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the supernatant which was used for further studies.

Protease assay

To 0.25 ml culture supernatant, 1.25 ml Tris buffer (100 mM; pH 9.0) and 0.5 ml 1% aqueous casein solution were added. The mixture was incubated for 30 min at 30°C. Then, 3 ml 5% trichloroacetic acid (TCA) was added to this mixture, whereby it formed a precipitate. The mixture was further incubated at 4°C for 10 min, and then centrifuged at 5,000 rpm for 15 min. Thereafter, 0.5 ml supernatant was taken, to which 2.5 ml 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. To this mixture, 0.5 ml folin phenol reagent was added and the absorbance was read at 660 nm using a UV Spectrophotometer. The amount of protease produced was measured with the help of a tyrosine standard graph (Takami et al., 1989).

Optimization for protease production

Effect of pH on protease production

The optimum pH for protease production was determined by adjusting the production medium to different pH values, for which pre-autoclaved medium was prepared individually at pH 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, and inoculated with experimental bacterium at 37°C.

Effect of temperature on protease production

Production medium at pH 9 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from 20, 30, 40, 50, 60, 70 and 80°C for 48. At the end of incubation period, the cell free culture filtrate is obtained and used as enzyme assay.

Effect of carbon sources on protease production

The effect of various carbon sources such as starch, glucose, maltose, lactose, xylose and fructose was examined in the production medium.

Effect of nitrogen sources on protease production

The different nitrogen sources like yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulphate were examined for their effect on protease production.

Effect of NaCl concentration on protease production

The basal media were supplied with different concentrations of NaCl (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) for the efficiency of maximum protease production.

Effect of metal ions on protease production

Influence of various metal ions on protease production was determined by incubating the medium with different metal ions such as $CaCl_2$, $MnCl_2$, $CuSO_4$, KCl, and $MgCl_2$ at a concentration of 0.2%.

RESULTS AND DISCUSSION

Microorganism

In the present study, a protease producing strain *B. subtilis* was isolated from sea water of Cuddalore coast, Tamil nadu, India. Morphological and biochemical characteristics of the strain revealed that it is a gram-positive, endospore-forming bacillus with catalase enzyme activity 16S rRNA gene sequence analysis confirmed the identity of the strain was submitted to NCBI as *B. subtilis* NS and based on the evolution distance and the phylogenetic tree, this strain was identified as *B. subtilis* and designated *B. subtilis* NS (GenBank accession no KF735656) (Figure 1).

Effect of pH on protease production

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the rate of bioprocessing are pH and temperature. In the present study, the effect

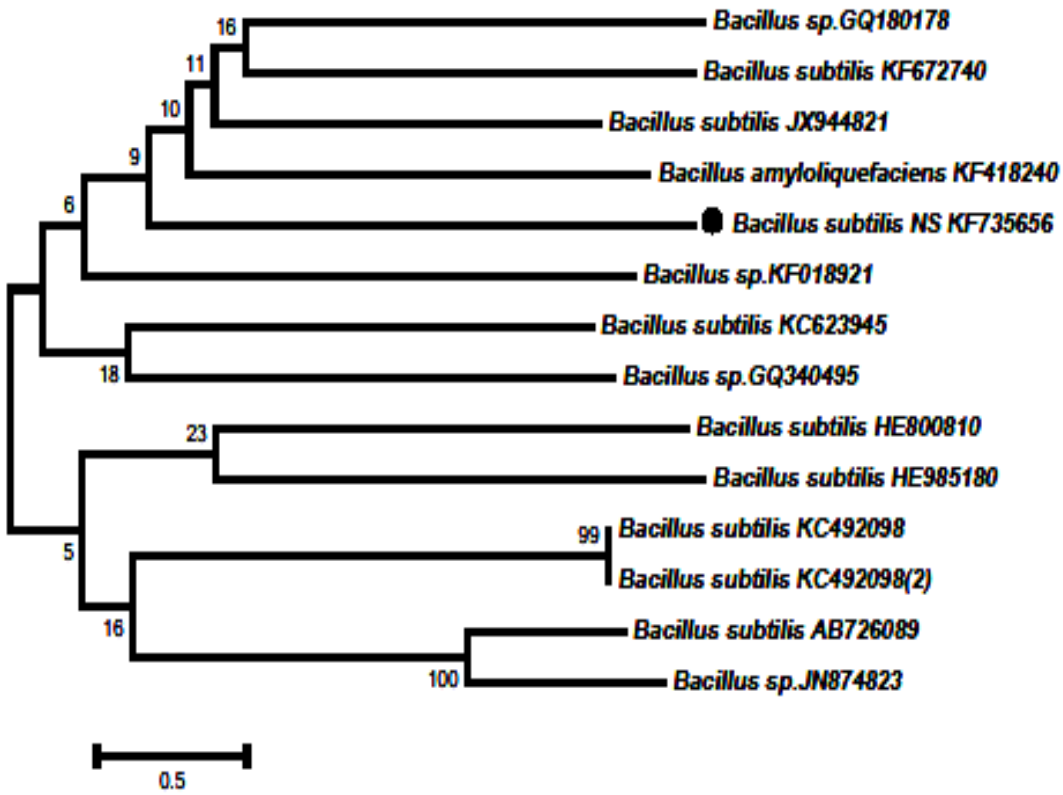

Figure 1. Phylogenetic tree of *Bacillus subtilis* NS strain 16S rRNA gene sequence with other *Bacillus* species.

of pH on protease production by *B. subtilis* NS revealed that pH 9 was optimal (123.5 U/ml) and enzyme production declined at the higher pH tested (Figure 2). This may be attributed to growth reduction and enzyme inactivation at higher pH (Tsujibo et al*.,* 1990; Mukesh Kumar et al*.,* 2012).

Effect of temperature on protease production

The effect of initial temperature on protease production showed that the higher protease production found at 40°C (117.4 U/ml) and minimum production (23.4 U/ml) was obtained in 80°C (Figure 3). The temperature influence enzyme production by changing the physical properties of the cell membrane. Usharani and Muthuraj (2010) were reported that protease production by *Bacillus laterosporous* was best at 37°C which indicates the same trend.

Effect of carbon source on protease activity

In the present study five different carbon sources were used for protease production. Since carbon is considered as the primary nutrient for the bacteria, different carbon source like sucrose, maltose, glucose, lactose, starch, fructose were analysed for the protease production. Maximum production of protease (199.01 U/ml) was observed in glucose when compare to other carbon sources (Figure 4). Maximum protease productions were obtained in xylose and maltose supplied medium by *Bacillus* sp. (Prakasham et al., 2006). Samarntarn et al. (1999) reported that protease production was high in the presence of supplementary carbohydrate carbon sources, especially lactose for microbes.

Effect of nitrogen source on protease production

The nitrogen source is important in fermentation media supplying a suitable nitrogen source favors higher level enzyme or metabolite production. In the present study, supplementary nitrogen sources accelerated protease production. Furthermore, this experiment showed that the complex organic nitrogen sources gave higher protease production than inorganic nitrogen sources, with production being highest in beef extract (118.42 U/ml)

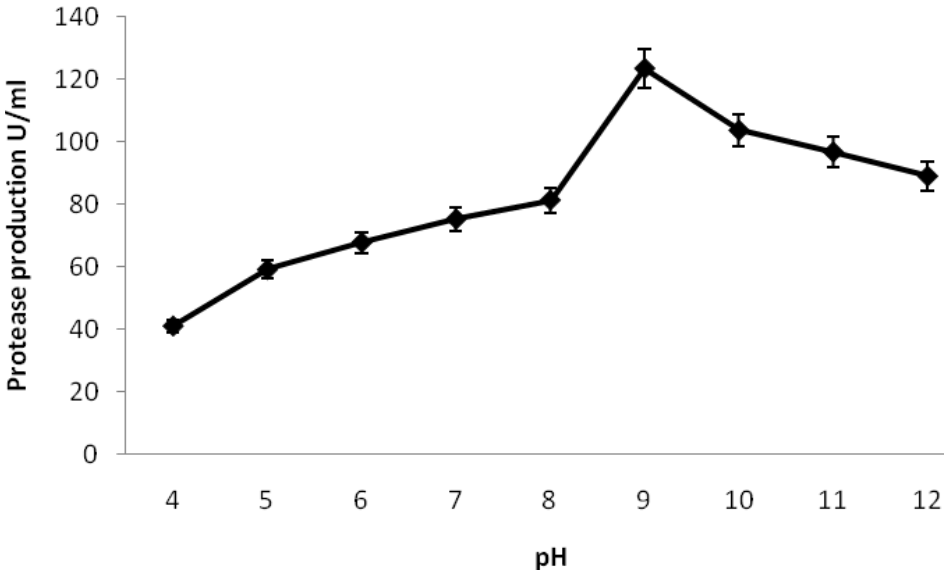

Figure 2. Effect of pH on protease production from *Bacillus subtilis* NS.

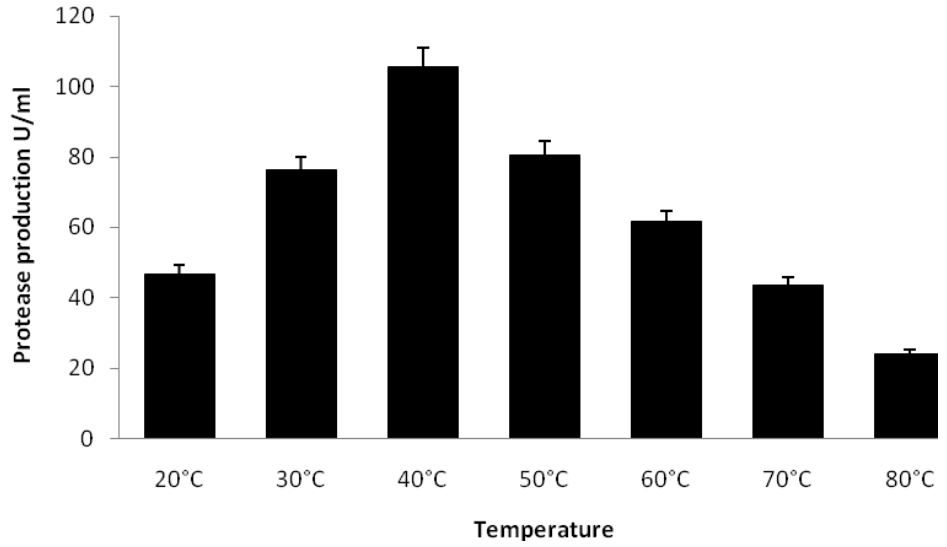

Figure 3. Effect of temperature on protease production from *Bacillus subtilis* NS.

supplied medium. Other organic nitrogen sources also support high protease production (Figure 5). It has been reported that organic nitrogen source like peptone, casein, yeast extract, favoured maximum protease production by *B. subtilis*. Next to that, inorganic nitrogen sources like ammonium carbonate followed by ammonium chloride, ammonium citrate and potassium nitrate were used as good nitrogen sources. Gupta et al. (2007) reported that the optimization of protease production in *Pseudomonas aeruginosa* PseA by using complex nitrogen sources. Our results also comply with the complex nitrogen sources induced protease production in *Aspergillus tamari* (Anandan et al., 2007).

Effect of metal ions on the protease production

The effect of various metal ions on protease production

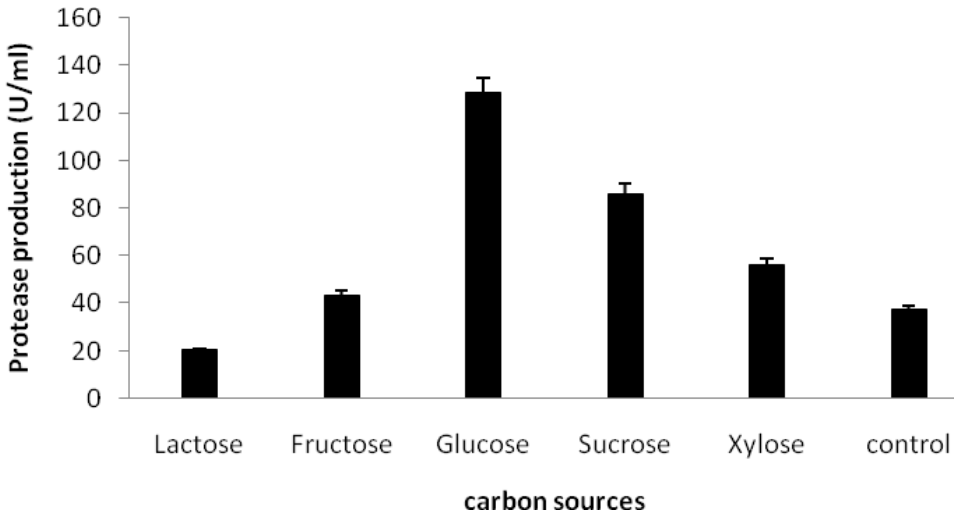

Figure 4. Effect of Carbon sources on the activity of protease enzyme from *Bacillus subtilis* NS.

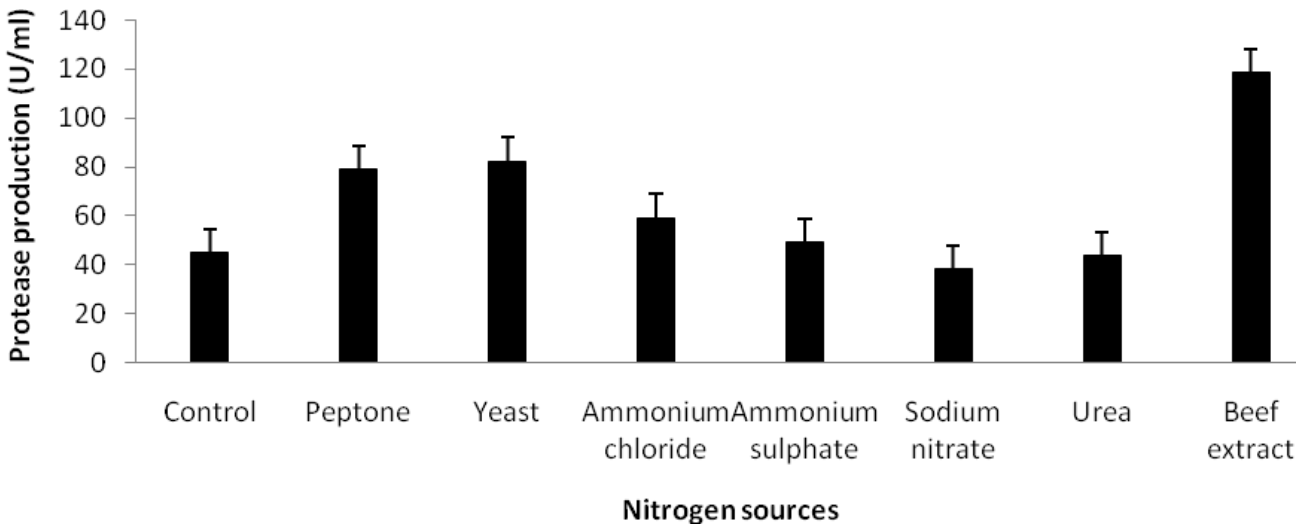

Figure 5. Effect of nitrogen sources on protease production from *Bacillus subtilis* NS.

was evaluated. Among these ions magnesium chloride was found to increase protease production of 149.29 U/ml (Figure 6). The enzyme activity was slightly enhanced by supplementation of K^+, Na^+ and Ca^+ ions compared to control. It was reported that supplementation of Mg^+, Ca_2^+ and K^+ salts to the culture medium exhibited slightly better production of protease. Rahman et al. (2005) observed that protease production was higher by *P. aeruginosa* in metal ions mediated culture. The present observation is in agreement with the earlier study reported by Krishnaveni et al. (2012) where the magnesium sulphate and manganese sulphate enriched medium enhanced the protease production in *B. subtilis.*

Effect of sodium chloride on the protease production

Regarding NaCl concentration, protease production was increased with increasing concentrations from 0 to 10% and reached its maximum at 7% (W/V) (Figure 7). There was a significant reduction in enzyme production found in the absence of NaCl (0%). The strain *B. subtilis* NS used in this study was isolated from marine water and that

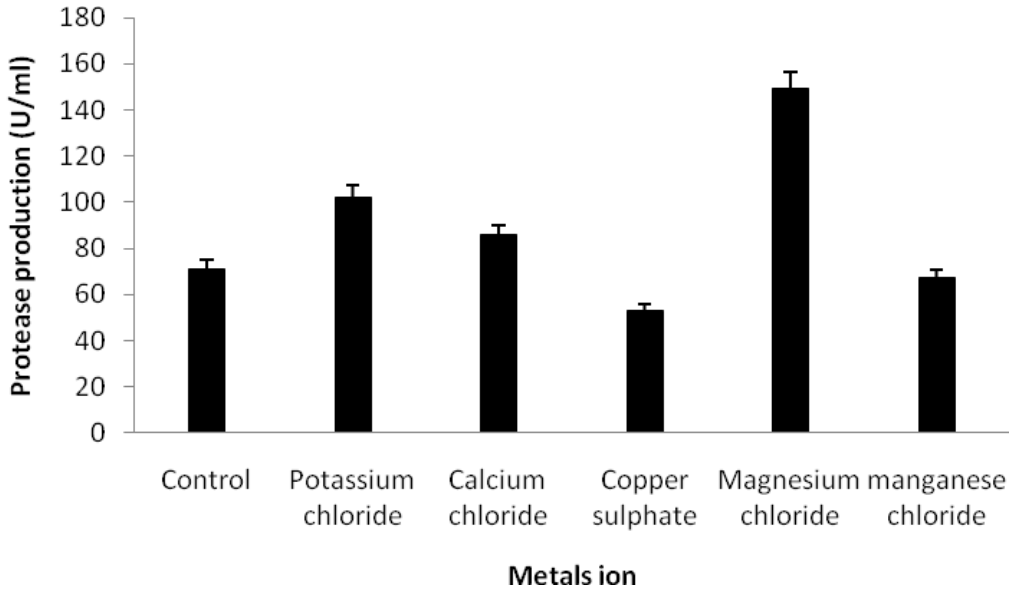

Figure 6. Effect of metal ion on the activity of protease enzyme from *Bacillus subtilis* NS.

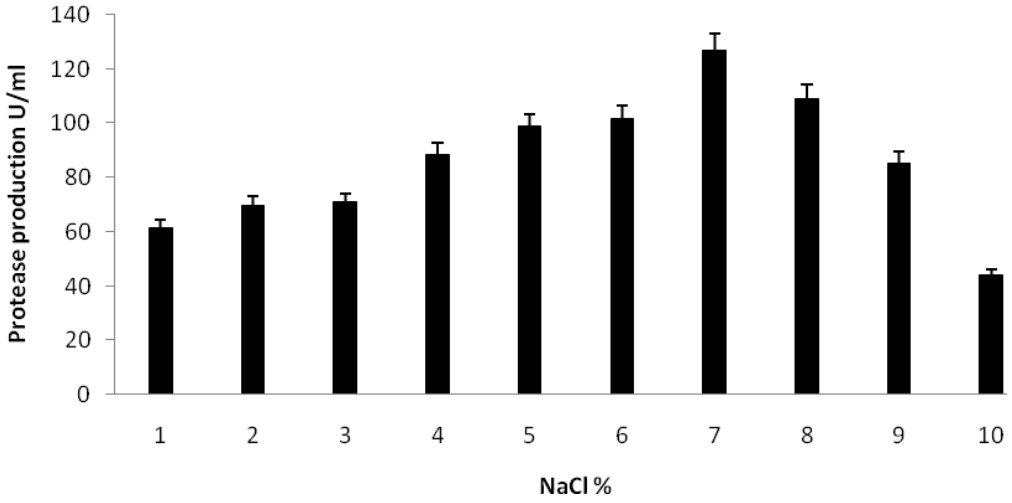

Figure 7. Effect of NaCl on protease production by *Bacillus subtilis* NS.

might be the reason for the higher protease production found at 30% and lesser at 0% NaCl. Similar results were observed in 1 M NaCl concentration by *Bacillus* sp. VITP4 isolated from Indian coastal area (Pooja and Jayaraman, 2009).

Conclusion

Proteases are industrially important enzymes with many applications, especially in the detergents industry. The enzyme from halophilic bacteria is an unexploited bio resource for enzyme production. The present study reports the production of protease by marine water isolate *B. subtilis* NS. Successfully, optimized environmental factors (pH and temperature) and nutrient (carbon, nitrogen, trace elements and sodium chloride) conditions yielded maximum protease production. This proteolytic bacterium could be used effectively for industrial purpose.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Growth of *Scenedesmus dimorphus* in different algal media and pH profile due to secreted metabolites

Ali Hussein Ali Al-Shatri[1], Ehsan Ali[3]*, Najeeb Kaid Nasser Al-Shorgani[2] and Mohd Sahaid Kalil[1]

[1]Department of Chemical and Process Engineering. Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia.
[2]School of Biosciences and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia, UKM, 43600 Bangi, Selangor, Malaysia.
[3]Centre for Energy Systems, National University of Sciences and Technology, Sector H-12, Islamabad Pakistan.

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In this study investigation was made to evaluate the effects of different algal media components to get optimized cell count of *Scenedesmus dimorphus*. Five different fresh water algal media such as Bold's Basal Medium (BBM), M4N medium, BG-11 medium, N-8 medium and M-8 medium were used for culturing *S. dimorphus* in flask culture. A set of environmental factors including light, temperature, air flow rate and nutritional components was standardized to obtain the highest productivity of 0.1406 g/L with specific growth rate of 0.10483/day. This study designates the bold basal medium as advantageous one for *S. dimorphus* and also reveals that production of metabolites by the same algal strain depends mostly on the nature of constituents of media and might have different influence on the pH.

Key words: *Scenedesmus dimorphus*, bold basal medium, algal growth.

INTRODUCTION

To meet the existing energy and environmental issues, renewable energy has been designated as a sustainable solution (Amin, 2009; Hallenbeck and Benemann, 2002). It has been documented that more than 80% of the energetic resources has been utilized to achieve the existing status of advancement on this planet (Huesemann, 2006). Algae as a source of oil/fuel has been documented as preferred on oil producing terrestrial crops. In addition to oil/fuel, algae are also well known for producing polyunsaturated fatty acids, which have been used as feed for fish and other animals (Harlioğlu, 2012; Narejo and Rahmatullah, 2010; Spolaore et al., 2006). Microalgae as a source of biofuel/renewable energy have been documented for significant environmental and commercial importance. Microalgae are not only sources of fuel, food for humans and animals, but are also the

*Corresponding author. E-mail: dr.ehsan@ces.nust.edu.pk. Tel: +92-51-9085 5275. Fax: +92-51-9085 5272.

sources of a wide range of chemical compounds used in industry, food technology, and pharmaceuticals as well (Bruton et al., 2009). Photosynthetic microalgae are potential candidates for utilizing excessive amount of CO_2, since these organisms are capable of fixing CO_2 to produce energy and chemical compound upon exposure to sunlight (Neenan et al., 1986). Microalgae have high growth rates and tolerance for varying environmental conditions. Microalgae can be grown in arid and in semi-arid regions with poor soil quality where woody or herbaceous crops cannot be grown. Saline water from aquifers or the ocean can be used for growing micro-algae. Such water has few competing uses and cannot be used for agriculture, forestry, or as potable water. The yield of biomass per hectare from microalgae is three to fivefold greater than the yield from typical crop plants (Bischoff and Bold, 1963).

Microalgae cultivation is gaining importance for its application in fuel and feed sectors in the world. A range of documented algal media can be used to cultivate specific algal strain for maximum growth and strategies can be designed to obtain the targeted products optimally. A number of algal media to culture freshwater algae have been reported, but the productivity is different from strain to strain. This study focuses on using five different media to culture fresh water algae *Scenedesmus dimorphus*. These media include Bold's Basal Medium (BBM), M4N medium, BG-11 medium, N-8 medium and M-8 medium (Eliasson et al., 1999; http://web.biosci.utexas.edu/utex/mediaDetail.aspx?mediaID=26). The source of carbon in all is carbon dioxide for photoautotrophic growth except BG-11 medium. The aim of this study is to determine the best medium for *S. dimorphus* in the batch photo bioreactors. Here, the study was designed to investigate the most suitable media under optimized influencing factors for the significant growth of *S. dimorphus* at lab scale. At the same time a critical investigation was made on nature of metabolites production from same strain but in different media with dissimilar nutrients or concentration of nutrients to influence the pH, a newly designed flocculant was also used to harvest the biomass for maximum biomass recovery.

MATERIALS AND METHODS

Growth media

All the chemicals used were of analytical grade unless pointed out clearly. Bold's Basal Medium (BBM) was prepared using distilled water, and the pH was adjusted (6.7 ± 0.3) with 5 N sodium hydroxide and 5 N hydrochloric acid (Bischoff and Bold 1963; Rowley, 2010). M4N medium and BG-11 Medium were prepared according to reported media recipes (Mandalam and Palsson, 1998) and N-8 and M-8 Media were prepared according to reported media recipes (Guillard and Ryther, 1962).

Algae strain

S. dimorphus is in the Chlorophyta family and was provided by Algaetech International Sdn Bhd Malaysia on agar Petri plates. Algae are cultivated first by transferring to test tube in BBM media. The test tubes are placed in a well-lit window with temperature 28 to 30°C until the medium turns green, signaling adequate algae growth. They were transferred to the batch PBR (Duran bottle 2 L size), where a larger volume (900 ml) of medium was used for higher biomass.

Growth of algal strains in batch system

The batch system, shown in Figure 1 was used for studying culture performance. In this system, 2 L Duran bottles were used as batch reactors and sealed with cap stoppers with two stainless tubes through which air was fed, exhausted and screwed by plastic cover. Air flow through polyvinylchloride pipes connected with 0.2 µm membrane filters and connected between stainless steel tubes and air pumps. The air feed tube was kept immersed inside the growth container and angled at the bottom of the container to allow mixing, to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, and to improve gas exchange between the culture medium and the air. Two cool white fluorescent lamps were employed as the light source of growth with an average light intensity of 1.3 ± 0.05 KLux. The volumetric flow rate of air was 1.5 L/min. Bioreactor temperature was monitored at ambient laboratory conditions as 30 ± 2°C.

Effect of different media on *S. dimorphus* growth rate

Cultures were subjected to five different media of different chemical compositions, BBM medium, M4N medium, GB-11 medium, N-8 medium and M-8 medium. All the media were in broth form, growth was monitored through optical density (OD) and cell count using microscope. Simultaneously, five batch photobioreactor (Scott Duran bottles) having 2 L capacity containing 900 mL of each medium and 100 mL *S. dimorphus* were subjected to evaluate the effect of different media on *S. dimorphus.* All media in the duran bottles were pre sterilized in autoclave at 121°C for 20 min before inoculation. The cultures were incubated at room temperature 30 ± 2°C under continuous light illuminated with cool fluorescent lamp (the light intensity was 1.3 ± 0.05 KLux). High quality chemical constituents were used for preparation of media with maximum accuracy (0.0001 g) in weighing by using Electronic balance, Precise, XT220A. Observations of algae growth was carried out daily in all media. The initial cell concentration was 5.3×10^6 cell/mL. To avoid settling, and for accelerating the growth process, air supply with constant volumetric flow rate 1.5 L/min (1.5 vvm) was used. Growth was followed through optical density during 15 days which was recorded by using spectrophotometer at wavelength of 730 nm.

Specific growth rate of *S. dimorphus*

Specific growth rate is a measure of number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture. The exponential (straight line) phase of growth was carefully determined and specific growth rate was obtained using Equation (1) (Chisti, 2007).

$$\mu = \ln(N_t/N_0)/T_t - T_0 \quad (1)$$

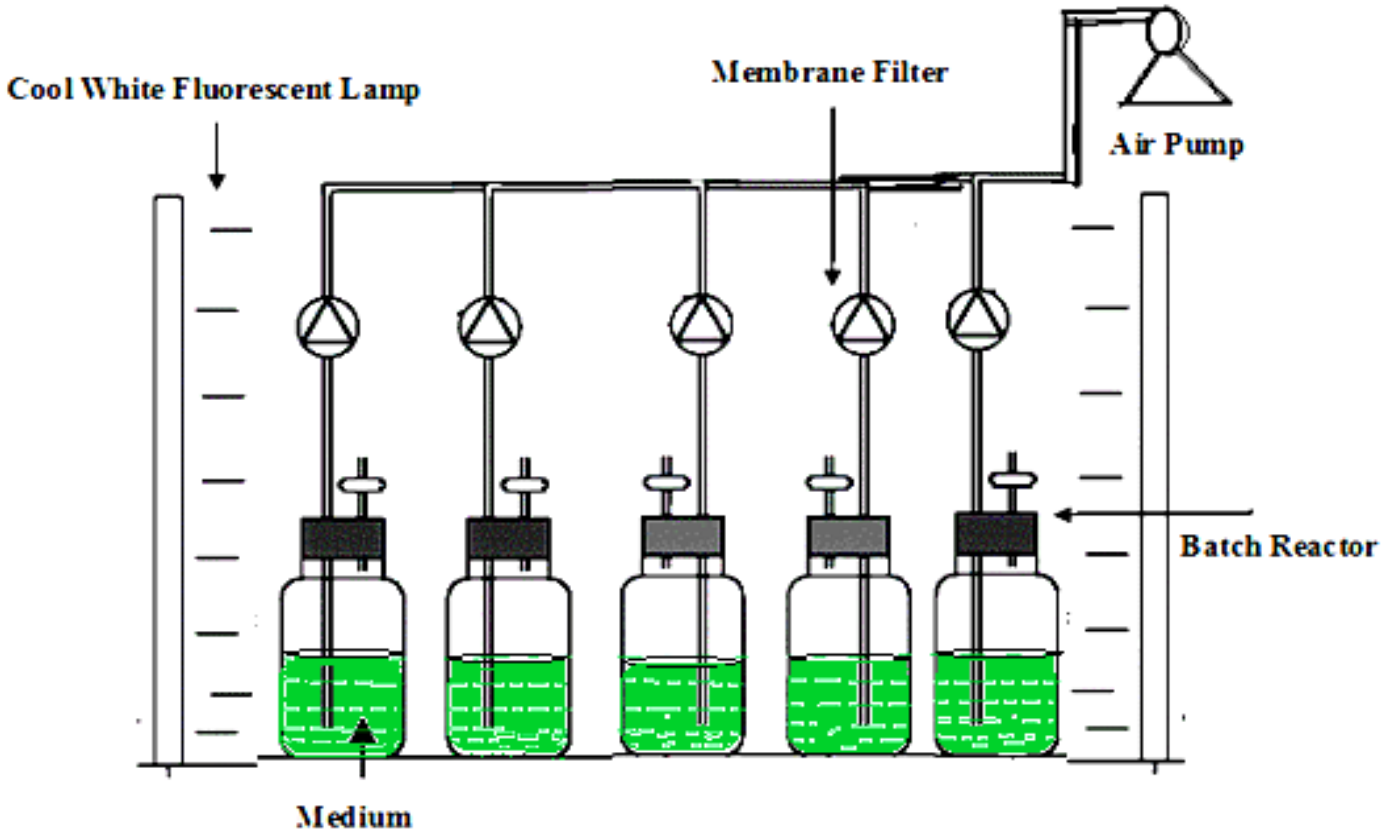

Figure 1. Schematic diagram of batch photobioreactor system.

N_o is number of cells at the start of log phase, N_t is number of cells at the end of log phase. T_o is Starting day of log phase, T_t is Final day of log phase.

CO_2 fixation (g/L.d)

Biomass and carbon production can be calculated based on fact that the microalgae contain 53.55% carbon (Reddy, 2002), some reports said micro algal biomass contains approximately 50% carbon by dry weight which presents that 1 kg of dry algal biomass utilize about 1.83 kg of CO_2 (Dragone et al., 2010; Reddy, 2002). The percentage of carbon fixed based on input can be calculated by using Equation (2).

$$C\ \% = \frac{\left(\frac{gC\ product}{day}\right)}{\left(\frac{gC\ input}{day}\right)} * 100 \quad (2)$$

Biomass recovery using a newly designed flocculant

A flocculant was prepared using palm oil industry waste (waste activated bleaching earth). The flocculant was in possession of aluminium and silica contents (unpublished data UKM, Biotechnology Lab). The composition of flocculant was two separate solutions of 350 ppm aluminum in 1N HCl and 400 ppm silicon in 1N NaOH. Different concentration of both parts of flocculant 1 to 5% (w/v) (with a unit difference of 1) were added together at room temperature (25 to 28°C) to each 500 ml beaker containing 400 ml of algal culture making both solutions finally neutralized, then biomass removal was monitored at 2, 4 and 6 h intervals.

In order to characterize harvesting of algal cells (as a result of coagulation/flocculation) by a new flocculant prepared in our laboratory as a solution form, the batch experiments were done using a six Scott duran bottles 250 mL filled with 200 mL of *S. dimorphus* collected after stationary phase. The duran bottles allow comparison of five different doses of flocculant to determine the required dose for adequate removal of suspended microalgae and the sixth bottle was used as a control. Doses used in these experiments to achieve removal of freshwater algae were 1, 2, 3, 4 and 5% (v/v), and the last one (control) without any flocculant.

In this process, it is essential that the flocculant was added by slow mixing to allow good contact between the small flocs and to agglomerate them into larger particles. The pH was measured before and during the flocculation period, also cell concentration and optical density at 730 nm was recorded. The biomass removal percentage was calculated during the flocculation by using Equation (3).

$$\%\ \text{Removal} = \frac{\text{Initial cell number} - \text{Final cell number}}{\text{Initial cell number}} * 100 \quad (3)$$

So, Equation (3) could be compacted to this formula.

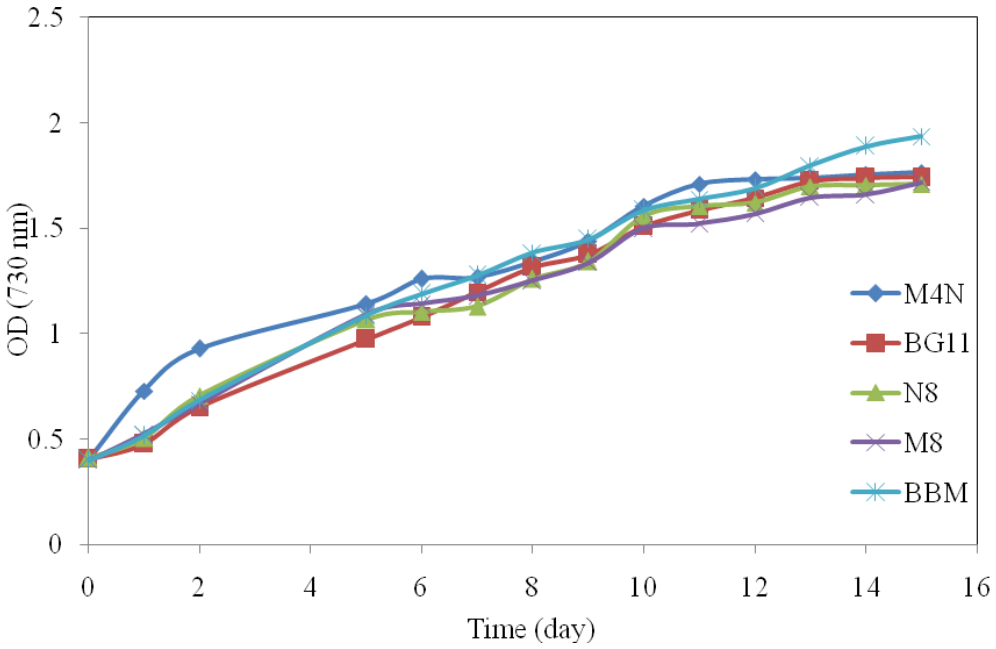

Figure 2. Growth of *S. dimorphus* in different media.

$$\% \text{ Removal } = \frac{\text{Cell removal}}{\text{Initial cell number}} * 100 \quad (4)$$

Cell removal efficiency percentage (%RE) was calculated using the following Equation (5) (Fogg, 1966).

$$\% \text{ RE} = \left[1 - \frac{\text{Final fluorescence}}{\text{Final fluorescence of control}}\right] * 100 \quad (5)$$

Where; Final fluorescence is optical density after flocculation process at limited time. Final fluorescence of control is optical density for the control sample at same time.

RESULTS AND DISCUSSION

Effect of different media on growth rate

Effect of different nutrient composition on growth pattern forms the objective of present work that aimed at selecting the best medium for *S. dimorphus*. Five runs with various culture media were carried out to select a suitable medium for cell growth of *S. dimorphus*. It can be seen in Figure 2 that *S. dimorphus* was growing faster (in 15 days) in Bold's Basal Medium (BBM) medium as compared to the other media. M4N and BG-11 came after BBM, while M-8 and N-8 came at the final as arranged. The growth curve did not show lag phase and it demonstrates that there was a quick adaptation of *S. dimorphus* to all media. pH was not controlled and the changes were monitored during the growth period as shown in Figure 3. Nature of metabolites secreted by the algae was not examined but their relation with pH was observed and evaluated. It was observed that M4N is the only medium which did not allow any abrupt change in pH and slowly increased the pH after 7th day of cultivation. In other words, algae in M4N medium were not secreting alkaline or acidic metabolites to affect the pH like algae in other media. *S. dimorphis* secreted alkaline metabolites in all other media and increased the pH during first five days and then got stable up to 15 days. This behaviour shows that the same strain can be influenced differently by different media constituent to produce metabolites of different nature.

The absorbance of the sample was measured using a spectrophotometer and correlated to calculate the dry weight or the number of cells per unit volume. The empirical equation for the calibration curve showed a linear relationship between optical densities and dry cell weight (g/L), represented by Equation (6). Also, a linear relationship between optical densities and biomass concentrations (cell*E+6/L) is represented by Equation (7).

$$y = 1.0885x \quad (6)$$

$$y = 13.055x \quad (7)$$

The biomass productivity, specific growth rate, optical density, cell concentration and CO_2 fixation were

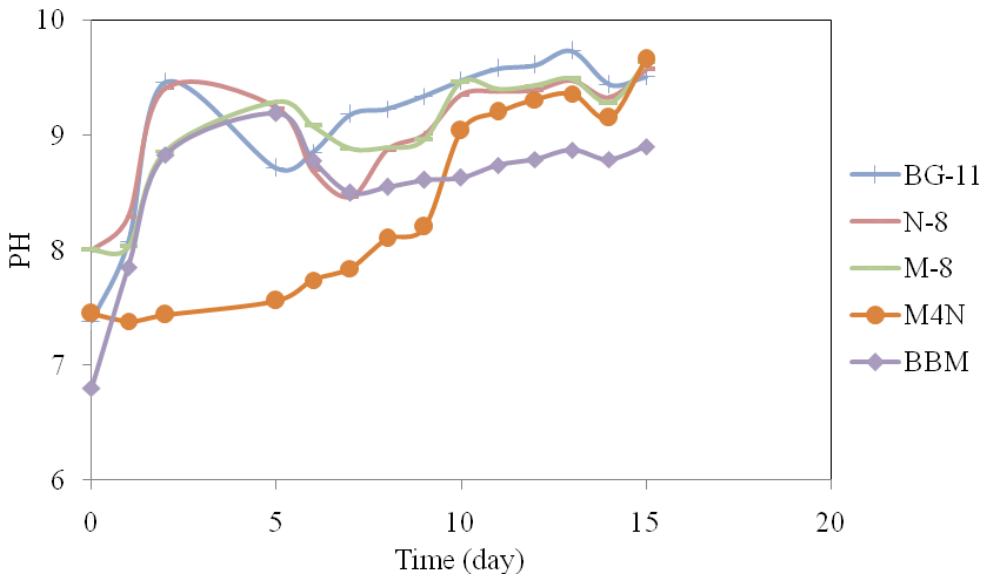

Figure 3. pH profile, while growing *S. dimorphus* in different media.

Table 1. Results of growth rate in culturing media.

Medium	BBM	M4N	BG11	N8	M8
Specific growth rate (μ)/day	0.10483	0.09781	0.09693	0.09492	0.09687
Final optical density (OD)	1.937	1.765	1.742	1.711	1.719
Final cell number (cell*E+6/L)	25.288	23.042	22.742	22.337	22.442
Biomass productivity (g/L.d)	0.1406	0.12808	0.1264	0.1242	0.1247
CO_2 fixation (g/L.d)	0.2573	0.2344	0.2313	0.2273	0.2282

estimated for all media as shown in Table 1, based on the equations (1), (6) and (7).The tabled values show the good comparison between all checked media which indicate that a bit better growth rate has been seen in the BBM medium.

The best result for growth of S*. dimorphus* was obtained when BBM was used (Table 1). The biomass productivity was 0.1406 g/L.d. Mass of C per day entering the system was 3.4339 g/day. Microalgae contain 50% carbon, so the amount of carbon production per day in the system was 0.0703 g/day. Thus by using equation (2), the percentage of carbon fixed based on input is:

$$C\,\% = \frac{\left(0.0703\ \frac{gC\ product}{day}\right)}{\left(3.4339\ \frac{gC\ input}{day}\right)} * 100 = 2.047\ \%$$

The theoretical carbon percentage found is less than 5 to 35% of the carbon fixed by marine phytoplankton and immediately lost from the cells as excreted organic matter (Yun et al., 1997). It may be due to high air flow rate used and most of carbon lifted from the system quickly with low dissolved amount, but it was best to keep culture on agitation and prevent our strain from settling. Reddy (2002) reported the carbon fixation based on the input 46.20 gC/day was approximately 3.65% for the flat-plate photobioreactor (Reddy, 2002). Yun et al. (1997) found that 0.624 g CO_2/L.d fixation from flue gas in wastewater medium at 15% (v/v) CO_2 was supplied to the air-adapted inoculum and at light intensity approximately 8 Klux, while in this study it was found 0.258 g CO_2/L.d in BBM with air only (Osborne, 2009).

Biomass recovery using a novel flocculant

The results for biomass recovery, removal percentage, and removal efficiency percentage are tabulated in Table 2. The results were in the trends to increase the biomass recovery by increasing the dose of flocculant from 1 to 5% (v/v). The maximal removal achieved at 5% (v/v) during 2 h, while more effective removal can be obtained

Table 2. Biomass recovery, biomass removal percentage and removal efficiency percentage at different doses during time.

Time (h)	2			4			6		
Flocculant (% v/v)	Cell (E+6/mL)	Removal (%)	Efficiency (%)	Cell (E+6/mL)	Removal (%)	Efficiency (%)	Cell (E+6/mL)	Removal (%)	Efficiency (%)
0	9.60	46.27	0.00	15.50	74.70	0.00	18.48	89.04	0.00
1	17.80	85.78	54.38	19.66	94.75	67.55	20.17	97.18	15.84
2	19.30	93.01	84.38	20.10	96.88	87.09	15.84	20.61	99.33
3	19.50	93.98	89.06	20.48	98.69	88.08	20.66	99.57	68.32
4	19.75	95.18	89.84	20.59	99.20	90.40	20.67	99.63	75.25
5	20.10	96.87	92.19	20.66	99.55	92.38	99.82	78.22	18.48

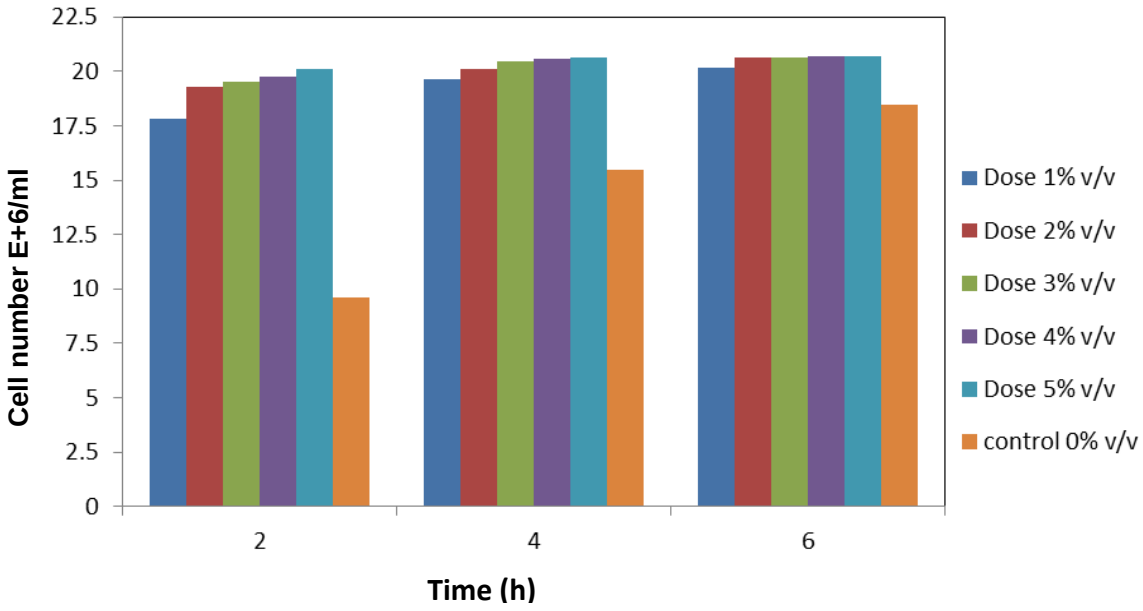

Figure 4. Biomass recovery during time with different doses of flocculant.

at 1% (v/v) by extending the period to 6 h as shown in Figures 4 and 5, where the initial cell concentration was $20.75*10^6$ cell/mL. The removal percentage was calculated by using Equation (4) and plotted per each dosage of flocculant. The results showed the maximal removal percentage that can be achieved at 5% (v/v) in 2 h (96.87%), but better removal can be achieved with 1% (v/v) dose after 6 h (97.18%) as shown in the Figure 5.

Also, the highest cell removal efficiency percentage (% RE) was obtained at dose 5% (v/v) 92.19% after 2 h, while the lowest (% RE) was obtained at dose 1% (v/v) 54.38% after 2 h.

Conclusion

The findings revealed that the Bold basal medium (BBM) is the optimal suitable medium for culturing *S. dimorphus* due to the comparatively high productivity of biomass (0.1406 g/L.d) at temperature 30°C, free air volumetric flow rate 1.5 L/min, and under continuous light intensity 1.3 ± 0.05 KLux.

Regarding metabolite production, some valuable findings can be expressed here as, the metabolite production from the same strain may vary to express different pH in different media. It was observed that the metabolites produced from *S. dimorphus* were mostly neutral in the beginning while growing in M4N but the other media presents some alkaline nature of metabolites during all growth period.

Variation in the pH of culture depends on the nature of metabolites secreted in response to the nutrients in different media but not due to the nature of algal strain.

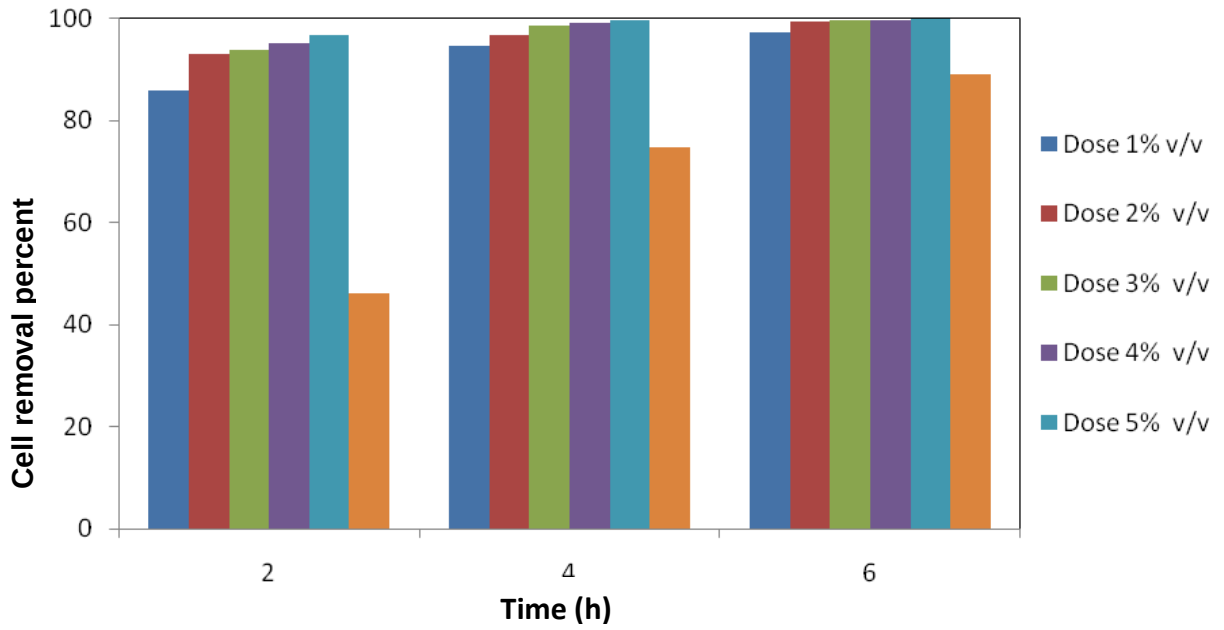

Figure 5. Removal percentage of biomass/algae using different doses of flocculant with time.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Control of lethal browning by using ascorbic acid on shoot tip cultures of a local *Musa* spp. (Banana) cv. Mzuzu in Tanzania

Munguatosha Ngomuo[1], Emerald Mneney[2] and Patric Ndakidemi[1*]

[1]School of Life Sciences, Nelson Mandela African Institute of Science and Technology, P.O. Box 447, Arusha, Tanzania.
[2]Mikocheni Agricultural Research Institute, P.O. Box 6226, Dar es salaam, Tanzania.

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The use of ascorbic acid during explants preparation and the effect of different concentrations of ascorbic acid in controlling lethal browning and survival of the explants in local banana cv. Mzuzu banana were investigated. The explants were taken from young suckers. The shoot tips were cultured on Murashige and Skoog's media supplemented with 5 mg/l of benzylaminopurine (BAP) and different concentrations of ascorbic acid (0, 50, 100 and 200 mg/l). Completely randomized design was used in this study. The results indicate that the use of ascorbic acid as an antioxidant during explants preparation significantly reduced the extent of lethal browning and survival of the explants followed by 100 mg/l of ascorbic acid applied directly into the media.

Key words: Micro propagation, surface sterilization, survival of explants, tissue culture.

INTRODUCTION

Banana contains constituents of phenolic enzymes principally polyphenoloxidase enzyme. Polyphenoloxidase enzymes serve as a very important phyto auxine in banana and help to defend the plant against infection from fungi, viruses and bacteria when injured (Chiremereze et al., 2011). The constituent of phenols in *Musa* spp. are principally dopamine, catechin, chlorogenic acid, cinnamic acid, hydroxyl benzoic, Resorcinol, progallic acid, salicylic acid, ferulic acid, vanillin coumurin, P-coumaric acid and phenol (Khalil et al., 2007). Browning reactions and astringency of the fruit caused by phenolic compounds are responsible for high mortality rate (lethal browning) in third generation of tissue culture. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds resulting in the formation of quinines which are highly reactive and toxic to plant tissue (Titov et al., 2006).

Apart from being an important group of secondary metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid (IAA) catabolism (Ozyigit et al., 2007). They also play effective

*Corresponding author. E-mail: ndakidemipa@gmail.com. Tel: +255757744772.

Abbreviations: MS, Murashige and Skoog (1962); **AA**, ascorbic acid; **BAP**, benzylaminopurine; **AC**, activated charcoal.

role in plant growth regulation, cell differentiation and organogenesis (Mato et al., 2006). Their concentration is often affected by several internal and external factors (North et al., 2011). Other factors include stress factors such as drought, water, radiation and pathogen infection from injured surfaces, which directly affects the concentrations of phenolics in plants (Kefeli et al., 2003).

Control of lethal browning in tissue culture of banana has been reported by different studies. Chimereze et al. (2011) reported the use of antioxidant potassium citrate and citrate (K-C:C) in prevention of browning in plantain culture. As an antioxidant, potassium citrate-citrate reduced browning within 2 h before culturing the tissues (Chimereze et al., 2011). Ascorbic acid is also an antioxidant used to control oxidation of phenols (Bharadwaj and Ramawat, 1993; Chawla, 2002; Abeyaratne and Lathiff, 2002). In controlling lethal browning in faba beans, Abdelwahd et al. (2008) reported that the use of activated charcoal, ascorbic acid, cystine and silver nitrate had a significant effect on the number of shoots and the length of shoots regenerated per explants. Strosse et al. (2004) reported that addition of cysteine to the growth media reduced explant blackening in banana tissue culture (Strosse et al., 2004). Understanding the processes contributing to the oxidation of phenols and how these can be minimized when initiating banana tissue culture is critical for successful *in vitro* culture, not only of banana but also of other crops.

Mzuzu variety is among the banana varieties susceptible to tissue browning and elimination or reduction of this process is necessary requirement for successful culture establishment. Development of suitable and efficient treatment to minimize tissue browning of *Mzuzu* variety was the objective of this study. The emphasis is particularly focused on the use suitable antioxidant concentration in the media and method of application during explant preparation.

MATERIALS AND METHODS

Plant materials

Young banana suckers of variety *Mzuzu* were collected from banana plantation grown at Chambezi outside Dar es salaam City near Bagamoyo.

Sterilization

The banana suckers were trimmed to remove extraneous matters and roots. They were then washed with tap water and a liquid detergent. The suckers were further trimmed and cut to a size of 10 cm by 5 cm to form explants which were soaked in a solution of distilled water and in a solution with concentration of 1.2 g/l of ascorbic acid. The explants were sterilized with 70% alcohol for 1 min and then rinsed three times with sterile distilled water. The explants soaked in 3.85% solution of sodium hypochlorite (NaOCl) for 1 h and two drops of tween 20. The explants were further sterilized with 1.925% solution of sodium hypochlorite. The explants were trimmed to remove the excess hypochlorite and aseptically placed on the culture media.

Culture conditions and media

The explants were placed in culture vessels containing 20 ml of culture media with MS basal salts supplemented with 20 g/l sucrose, vitamins glycine 2 g/l, pyridoxine 0.5 g/l, Nicotinic acid 0.5 g/l, Thiamine 0.1 g/l and Myo inositol at 0.1 g/l. The media will also be supplemented with 5 mg/l of BAP and solidified with 4.0 g/l of agar. The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min.

The experiment consisting of five treatments of ascorbic acid (0 mg/l, 0 mg/l but soaked in 1.2 g/l of ascorbic for 1 h during explants preparation, 50, 100 and 200 mg/l) investigated the use of ascorbic acid in explants preparation and the effects of ascorbic acid concentration in controlling lethal browning during culture initiation of banana. Five culture vessels were used for each treatment; each culture vessel contained one explant and each treatment was replicated three times.

Data collection and analysis

Data on number of healthy and growing plantlets and numbers of diseased plantlets were collected on weekly intervals for a period of four weeks after initiation. Based on visual observation, the extent of media discoloration was assessed. Media discoloration was rated on scale of 1 to 4 (1 implies no discoloration, 4 implies extreme media discoloration). Data collected were analyzed for statistical significance using analysis of variance (ANOVA). These computations were done by using a statistical software program STATISTICA software Programme version 2013 (StatSoft Inc., Tulsa, OK, USA). Fisher least significance was used to compare means at P = 0.05 level of significance.

RESULTS AND DISCUSSION

Effect of various concentrations of ascorbic acid in controlling the extent of lethal browning

In this study, the extent of browning and death of explants was observed on weekly basis for four weeks. Generally, lethal browning increased with time, but decreased with increased concentration of ascorbic acid and then declined at highest concentration (Table 1). During this experiment, highest degree of lethal browning was observed in control treatment (Table 1). The mortality of explants was also high in the control treatment (Table 2) and (Figure 1). It is well established that injured tissues normally stimulate the production of phenols (Dodds and Roberts, 1995). This is a defensive mechanism common in plants in response to tissue damage (Pan and van Staden, 1998; Ndakidemi and Dakora, 2003). The production of these compounds in excess results in browning and eventually death of explant (Figure 1). The darkening or browning of the media in tissue culture is caused by exudation and oxidation of phenollic compounds which results in the formation of quinones which are highly reactive and toxic to plant tissues (Ko et al., 2009).

In this experiment, the use of ascorbic acid during

Table 1. Extent of media discoloration after four weeks.

Treatment	Time (week)			
	1	2	3	4
Concentration				
0 mg.l^{-1} A.A	3.00 ± 0.33^{a}	3.40±0.2^{a}	3.60±0.16^{a}	3.70±0.15^{a}
0 mg.l^{-1} washed with A.A	1.50 ± 0.20^{b}	1.93±0.3^{b}	2.00±0.33^{b}	2.00±0.33^{c}
50 mg.l^{-1} A.A	1.91 ± 0.31^{b}	2.63±0.36^{ab}	2.63±0.36^{ab}	3.09±0.34^{ab}
100 mg.l^{-1} A.A	1.53 ± 0.21^{b}	2.07±0.34^{b}	2.07±0.34^{b}	2.13±0.36^{c}
200 mg.l^{-1} A.A	1.23 ± 0.21^{b}	2.31±0.38^{b}	2.23±0.38^{b}	2.31±0.36^{bc}
One way ANOVA (F- Statistic)				
Main effect				
Concentration	7.52**	2.63*	3.31*	4.17*

*, P≤0.05; **, P≤0.001; ns, not significantly different. Values (Mean ± SE) followed by dissimilar letters in a column are significantly different by Least significant difference (LSD) test at P=0.05. The rating scale is 1 to 4 (1, No media discoloration; 4, extreme discoloration).

Figure 1. Dead explant due to browning (the picture was taken at the fourth week of the experiment).

explants preparation showed the best results in controlling lethal browning throughout the experimental period. The analysis indicated that there were significant ($p \leq 0.05$) differences in the extent of lethal browning in control treatment (3.70) relative to the use of ascorbic acid (2.0) during explants preparation (Table 1). The successful use of antioxidant applied during explants preparation to prevent lethal browning is also reported by Titov et al. (2006), in which an antioxidant wash of 0.125% potassium citrate:citrate (K-C:C in a ratio of 4:1 w/w) solution was useful for explants preparation of *Musa* spp.cv. Kanthali (Titov et al., 2006).

Good control of lethal browning in terms of concentration of ascorbic acid applied on the media was observed at the concentration of 100 mg/l followed by the concentration of 200 mg/l (Table 1). At the concentration of 100 mg/l, ascorbic acid significantly reduced the extent of lethal browning on the explants at fourth week compared with the rest of other treatments. Similar to our study, Strosse et al. (2004) indicated that antioxidants such as ascorbic acid or citric acid in concentrations ranging from 10 to 150 mg/l added to the media reduced browning in banana varieties.

The wide range in concentration of antioxidant added to the media to control browning is due to the fact that the extent of lethal browning is genotype specific as it depends on the cultivar or variety. In our study, ascorbic acid at concentrations of 100 and 200 mg/l showed good control

Table 2. The effect of ascorbic acid concentration on survival of explants.

Concentration	Number of explants	Total number of dead explants on weekly basis [Time (weeks)]				Percentage of surviving explants after 4 weeks
		1	2	3	4	
0 mg.l^{-1} A.A	10	4	5	7	10	0
0 mg.l^{-1} A.A, soaked in A.A	14	0	0	4	5	64.3
50 mg.l^{-1} A.A	11	1	2	7	9	18.2
100 mg.l^{-1} A.A	15	0	4	7	7	53.3
200 mg.l^{-1} A.A	13	0	3	6	6	53.8

Figure 2. Surviving explant due to application of ascorbic acid.

of lethal browning.

During the first week of this experiment all treatments were significantly ($p \leq 0.001$) not different except for a control which was significantly different to the rest of the treatments. This indicates that even at low concentration of 50 mg/l, ascorbic acid was able to control lethal browning at least in the first week. Ko et al. (2008) reported similar results where a low concentration of ascorbic acid (0.0005%) applied directly on the surface of the media after autoclaving was able to reduce the number of diseased plantlets per flask from 10.7 without ascorbic acid to 4 and increased the number of healthy plantlets from 1.7 in control to 15.0. However, from second week, the low concentration of ascorbic acid was not significantly different to the control (Table 1).

Effect of various concentrations of ascorbic acid on survival of explants

The death of explants due to lethal browning was mostly observed in the control treatment. Generally, the highest death of explants was observed on third and fourth week of the experiment (Table 2). The observed death of the explants with time was attributed to the oxidation of polyphenolic compounds released from the wounded tissues which formed the barrier round the tissues preventing nutrient uptake and hindering growth (Strosse et al., 2004). The death pattern of these explants was similar to the extent of browning in the respective treatments. The lowest survival of explants was observed in the control treatment where there was no explant which survived (Table 2). The highest survival of explants was observed in treatment of soaking the explants for 1 h in 1.2 g/l of ascorbic acid during explants preparation. About 64.3% of the explants survived the incidence of lethal browning (Figure 2). This was followed by the use of ascorbic acid at concentration of 200 mg/l applied directly to the media where 53.8% of the explants survived (Table 2).

Conclusion

This study indicated that lethal browning in cv. Mzuzu can be controlled by the use of ascorbic acid during explants

preparation. Treating the explants with 1.2 g/l of ascorbic during explants preparation and addition of 100 mg/l in the growth medium controlled the extent of lethal browning of the explants significantly compared with the rest of different concentrations of ascorbic acid added to the media after four weeks of experimentation. In order to minimize the cost of tissue culture and the losses associated with death of explants, it is recommended that the use of ascorbic acid as an antioxidant can be applied during explants preparation to avoid addition directly to the media which might cause unforeseen problems to nutrient absorption and general availability of nutrients. It can be concluded that application of ascorbic acid directly to the media at appropriate concentration can control lethal browning in this variety but high concentration can be deleterious to the explants, while low concentration might be ineffective. To produce an optimized culture with low mortality of explants, ascorbic acid should be applied before surface sterilization.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Concentration of fecal corticosterone metabolites in dominant versus subordinate buffalo heifers

Aparecida F. Madella-Oliveira[1], Celia R. Quirino[2]*, Aline Pacheco[2], Ricardo L. D. da Costa[3], Renato T. Beltrame[4], Weliton M. Costa[5], Cláudio A. de Oliveira[6] and Priscila V. Furtado[6]

[1]Instituto Federal do Espírito Santo – Campus de Alegre – ES, Brazil.
[2]Laboratório de Reprodução e Melhoramento Genético Animal, CCTA, Universidade Estadual do Norte Fluminense Darcy Ribeiro-RJ, Brazil.
[3]Instituto de Zootencia, da Agência Paulista de Tecnologia dos Agronegócios, Rua Heitor Penteado, 56, Nova Odessa, São Paulo, Brazil.
[4]Centro Universitário do Espírito Santo-UNESC; Av. Fioravante Rossi, 2930, Colatina, ES, Brazil.
[5]Graduate Student of Instituto Federal do Espírito Santo – Campus de Alegre – ES, Brazil.
[6]Hormone Dose Lab of Universidade de São Paulo- SP, Brazil.

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The objective of this work was to evaluate the concentration of fecal metabolites of corticosterone and to verify if there are differences between dominant and subordinate heifers. The feces of 18 buffalo heifers were collected in the estrous period, to quantify the corticosterone concentrations. The heifers were separated into three groups (G1, G2 and G3) and synchronized. The observations of the social and sexual behaviors were recorded and, from these results, the sociometric matrix was constructed to establish the social index and determine the hierarchic positions of the buffalo heifers as low, moderate and high. The fecal concentrations of corticosterone were higher in animals with high hierarchic position on day zero and describe alterations in the dominant females before synchronization, suggesting that there is an energy cost for the females in the highest position to be able to maintain their dominance status.

Key words: Estrus, hormones, social status, non-invasive technique.

INTRODUCTION

Several studies have been conducted linking glucocorticoids to social and sexual behavior of animals. Glucocorticoids (cortisol and corticosterone) have been widely researched. Among the main functions of these hormones can be highlighted as the role in the adaptive response that occurs during the stress process. For the buffalo species, there are some studies addressing glucocorticoid hormones (Prakash and Madan, 1984; Napolitano et al., 2004; Khan et al., 2011; Titaporn Khongdel et al., 2011).

The animals that live in social groups establish dominance-subordination relations through agonistic and

*Corresponding author. E-mail: crqster@gmail.com.

Table 1. Groups of buffalo heifers used in the social and sexual behavior test.

Group	Animal	Month of synchronization
G1	4, 6, 8, 10, 11, 14 and 24	April
G2	5, 7, 13, 15, 16, 18 and 20	May
G3	3, 9, 21 and 22	June

Table 2. Sexual and social behavior standards of buffalo heifers and the bull.

Behavior	Exhibited feature
Sexual behavior	Head with head, homosexual behavior mating with penetration, mating without penetration, attempt to mount, head in the haunch, follow the female and conduct flehmen
Social behavior	
Agonistic reactions	Push, expel
Non-agonistic reactions	Smell, lick and rub.

submission meetings that provide an increase in the release of glucocorticoids (Solano et al., 2004). Hasegawa et al. (1997) and Solano et al. (2004) verified that the concentration of cortisol influences the social behavior of cattle and detected differences in the concentrations of cortisol in plasma in relation to the hierarchic positions. However, monitoring of the concentration of glucocorticoids in the plasma can be influenced by the high level of stress caused during the blood sampling. For this interference not to occur, the non-invasive monitoring technique using feces is used, so as not to cause any stress during collection of samples. The technique of non-invasive monitoring has enabled the measurement of fecal metabolites of steroids with significant correlations with the concentrations of these hormones in the plasma and milk. It has been used mainly for researches that are addressing behavioral and reproductive including investigations of diurnal and seasonal patterns of cortisol level, social and dominance interactions, the impacts of habitat degradation, transport stress, predator-pre interactions and the effects of maternal stress (Schwarzenberger et al., 1996; Möstl and Palme, 2002; Sheriff et al., 2009).

Thus, the aim of this work was to evaluate the concentration of fecal metabolites of corticosterone and to verify if there are differences between dominant and subordinate between buffalo heifers.

MATERIALS AND METHODS

Experiment locale

The experimental work was conducted in the Cattle Rearing Sector of Instituto Federal do Espírito Santo, Campus de Alegre, Espírito Santo State, Brazil. The Campus de Alegre is located in south latitude 20°45'29" and west longitude 41°27'32", at an altitude of approximately 120 m.

Animals

A total of 18 heifers crossbred Murrah and Mediterranean animals were included in the study. The female were 20 months old and weighed an average of 300 kg. The experiment started in the month of April during the mating season for the buffalo species in the southeastern region of Brazil. The females were synchronized using the following protocol: On day zero, the animals received a Progesterone device (CIDR - controlled internal drug release intravaginal implant containing 1.9 g of progesterone) and 2 ml of Estradiol Benzoate was applied and, nine days later, the implant was removed and 2 ml of Prostaglandin and 400 IU of eCG (Equine chorionic gonadotropin) was applied

Sexual and social behavior

The heifers were divided into three groups (Table 1), being selected according to the preliminary observations of social behavior conducted in the pasture (Madella-Oliveira et al., 2012). The social and sexual behavior observations occurred during the induced estrus on day 9 of the synchronization, in the presence of the small bull. All social and sexual behavioral interactions were recorded continuously for five consecutive days, 24 h a day. Four observers worked in shifts of 6 h/day, equipped with binoculars and recorders, totaling 360 h. During the day, the observations were made in the pasture and at night inside the pen to facilitate recording of observations. The following behaviors, shown in Table 2, were recorded.

Collection and extraction of fecal samples for hormone dosage

The feces of 18 buffalo heifers were collected for analysis of corticosterone metabolites. The days of collection are shown in Table 3. The feces of the animals were collected fresh, and placed in previously identified hermetic plastic bags (animal number, date and time). After collection, the material was refrigerated at 4°C in Styrofoam containing recyclable ice and later frozen (-20°C). The

Table 3. Collections of feces for quantification of the fecal metabolite concentrations of corticosterone of buffalo heifers.

Parameter	Days of Collection	Time of collection (h)
Synchronized heat	Day zero*	8 to 10 am
	Day of estrus observation	At the time of heat identification.
	Day after estrus	12 to 24 h after collection on the day of heat observation.
	Fifth after estrus	8 to 10 am

Table 4. Means and respective standard deviations of the concentrations of fecal metabolites of corticosterone (ng/g) in relation to the groups (G1, G2 and G3) on day zero (D0), on the day of estrus (DESTRUS), on the day after heat (DAESTRUS) and on the fifth day after heat (FDAESTRUS).

Hormone	Group	Mean ± Standard deviation			
		D0	DESTRUS	DAESTRUS	FDAESTRUS
Corticosterone	G1	2,332.42 ± 2,138.33	1,629.57 ± 715.54	1,060.42 ± 547.57	2,629.85 ± 2,051.50
	G2	3,583.00 ± 3,514.76	2,182.00 ± 1,353.36	1,243.50 ± 447.26	2,370.85 ± 2,338.46
	G3	1,716.25 ± 239.73	2,908.00 ± 3,233.93	893.66± 217.78	2,723.50 ± 1,764.99

samples were kept at this temperature until processing of its extract. The procedure used to extract the fecal metabolites of corticosterone was conducted according to Graham et al. (2001). Aliquots of approximately 0.5 g (0.48 to 0.52 g) of feces were placed in previously identified glass test tubes (16 x 125 mm), to which 5 ml of 80% methanol was added. Later, the tubes were agitated for 30 s by vortexing followed by 12 h in a blood homogenizer. All the tubes were centrifuged at 1500 rpm for 15 min, and the supernatants were transferred to 1.5 mL Eppendorf tubes. The extracts were kept until quantification of the fecal corticosterone. ImmuChemTM corticosterone double antibody RIA kit (MP Biomedicals, LLC, Diagnostics Division, NY, USA) was used to quantify fecal corticosterone metabolites.

For the corticosterone analyses, the samples were diluted in proportions that varied from 1/10 to 1/40 in a steroid diluter of the kit (Phosphosaline gelatin buffer - pH 7.0, containing gamma globulin of rats), and hormone quantification was then conducted by RIA, in a Gamma radiation counter (Packard Cobra Auto-Gamma®), verifying the number of counts per minute (cpm). The results were obtained in ng/mL (nanogram per milliliter). The final metabolite values for corticosterone were converted to the weight and dilution used, through the formula below, to be expressed in ng/g (nanogram per gram of feces).

$$X = (C \times V \times D) / W$$

Where:

C = is the concentration in ng/mL provided by the test;
V = is the total volume of the extract, that is, quantity of solvent that was used to make the extraction (5 mL);
D = dilution of the extract that was used for the test;
W = is the weight of feces used in the extraction (usually between 0.1 and 0.5 g).

Calculation of the sociometric measures and statistical analysis

The results of the social behavior were transformed into a sociometric matrix, initiators of agonistic behavior are put in lines and receivers of these behaviors are put in columns. The social index was calculated according to Orihuela and Galina (1997). From the results of the social index, the heifers were classified into three categories according to the social index (SI) values: low (SI = 0.0 to 0.33), moderate (SI = 0.33 to 0.66) and high hierarchy (SI = 0.67). To evaluate the means of the concentrations of corticosterone metabolites, the GLM process was used (SAS, 2001) and the means were compared by the SNK test, 5% probability.

RESULTS

The mean concentrations of the fecal metabolites of corticosterone of the buffalo heifers were 2,681.83 ± 2566.72; 2,128.50 ± 1708.62; 1,097.81 ± 458.71; 2,549.94 ± 1,997.03 in ng/g of feces for day zero, on the day of estrus, on the day after estrus and on the fifth day after estrus, respectively. Table 4 shows that the means and respective standard deviations of the concentrations of the fecal metabolites of corticosterone among the groups (G1, G2 and G3) do not present differences. The corticosterone concentrations in relation to the hierarchic positions showed in Figure 1, differ statistically ($P<0.05$). The animals with high hierarchic position presented means higher than those of the other hierarchic positions (moderate and low), in relation to day zero (start day of synchronization). However, on the day of estrus, on the day after estrus and on the fifth day after estrus, no differences were found ($P>0.05$) in the concentrations of fecal metabolites of corticosterone among the hierarchic positions of the buffalo heifers.

DISCUSSION

In response to synchronization of the estrus cycle, all the buffalo heifers showed symptoms characterizing estrus, as the females started displaying estrus behavior 12 to 72 h after removal of the CIDR. The results showed that the concentration of fecal metabolites of corticosterone was low one day after estrus, which could suggest that

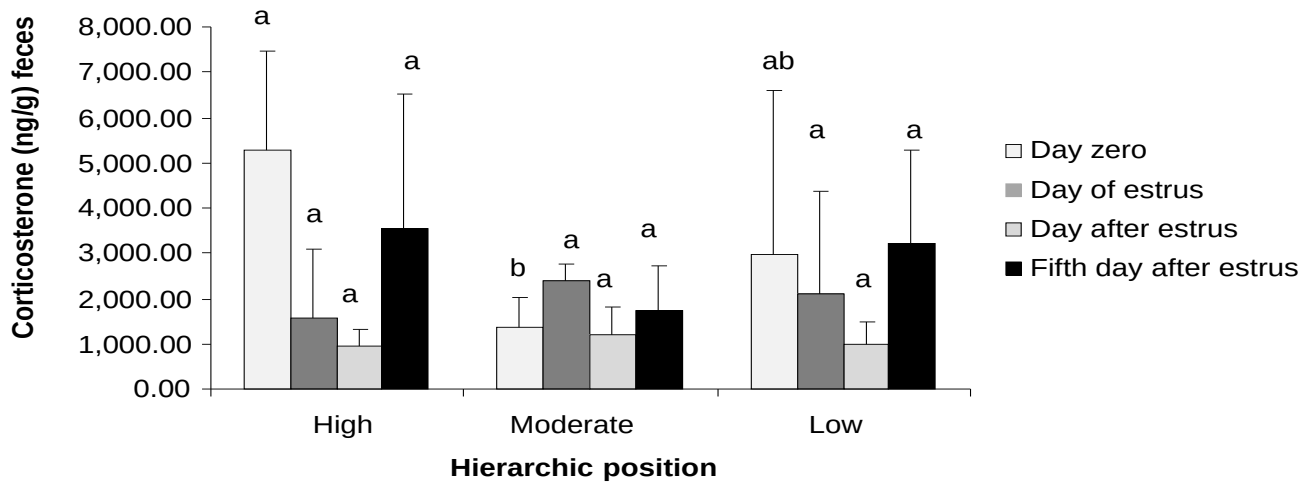

Figure 1. Mean and respective standard deviation of the concentrations of fecal corticosterone metabolites (ng/g) in relation to hierarchic positions of buffalo heifers on day zero, the day of estrus, the day after the heat and on the fifth day after estrus.

the bull's presence reduces the level of stress of the buffalo heifers. On day zero, when the heifers were not in contact with the bull and which was before the induced estrus, higher and significant values of corticosterone concentrations were detected in the heifers with higher hierarchic position, indicating that in the bull's absence, the animals with high hierarchic position showed a higher level of excitability. Making a comparison with the results of the plasma concentrations of cortisol in cattle, Solano et al. (2004) verified that cortisol influences the hierarchic behavior of cows and observed that the animals with greater dominance showed higher levels of cortisol, which would agree with results of the concentrations of fecal metabolites of corticosterone in the buffalo heifers. Encarnação (1983) and Hasegawa et al. (1997) observed low concentrations of glucocorticoids in the blood of dominant animals and higher concentrations as the position dropped in the social scale of the herd, in which the last animal had the highest stress.

Considering the results of this work, we verify the need for more studies on the social and sexual behavior of heifers, as well as determination of the fecal metabolite concentrations of glucocorticoids hormones in this species. There is no data published in the literature consulted that relates to fecal corticosteroid metabolites in heifers and social behavior, thus this study probably pioneers work of this kind in this species.

Conclusion

The results found for corticosterone indicate that only on the day before estrus was the stress level increasing, especially in the animals of greater dominance, in relation to the hierarchic position, suggesting that despite all the benefits of a dominant animal in relation to the subordinates, there are differential energy cost between high and moderate. The male's presence could indicate a reduction in the level of stress in the buffalo heifers.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Inhibition of carbon disulfide on bio-desulfurization in the process of gases purification

Ziyu Song[1, 2], Qiang Li[1], Dan Wang[3] Hang Tang[1, 2], Maohua Yang[1] and Jianmin Xing[1]*

[1]National key Laboratory of Biochemical Engineering, Institute of process Engineering, Chinese Academy of Sciences, Beijing 100190, People's Republic of China.
[2]Graduate University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China.
[3]College of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400044, People's Republic of China.

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Biological desulfurization is a novel technology for the removal of hydrogen sulfide from some biogas or sour gas, in which there are always a certain amounts of carbon disulfide together with much hydrogen sulfide. Nowadays, carbon disulfide is found to have negative effect on the biological desulfurization, but seldom research is afforded to investigate how carbon disulfide inhibits the process of biological desulfurization. In this paper, we investigated the effect of carbon disulfide both on the growth of *Thiobacillus thioparus* and the resting cells under various concentrations, including 0.01, 0.05, 0.10, 0.15 and 0.20%. In this process, the rate of the cell growth was characterized by the rate of nitrogen consumption in order to solve the problem that the adsorption of cells to sulfur granules have on the accuracy of biomass test. Under the cell density of 23.92 mg N/L, which is lower than the maximum of cell density 36.13 mg N/L, the average rate of thiosulfate oxidation reached the maximum (26.50 $S_2O_3^{2-}$ mg/L-h). Carbon disulfide at titers of 0.01% significantly inhibited the growth of cells, but hardly affected the biological desulfurization of resting cells. Although carbon disulfide at titers of 0.05% had negative effect on the biological desulfurization of resting cells, the effect of inhibition could be relieved by the increased density of resting cells. For the resting cells, the parameters of Michaelis-Menten equation were calculated by the method of Lineweaver-Burk. The V_{max} of biological desulfurization was decreased from 27.93 to 14.0 $S_2O_3^{2-}$ mg/L-h, and the K_m was increased from 0.264 to 0.884 mM, with the concentration of carbon disulfide rising up from 0.0 to 0.1%. These results show that the growth of cells was sensitive to carbon disulfide, and the resting cells had resistance to the low level of carbon disulfide (0.05%). Thus, the inhibition of carbon disulfide to biological desulfurization should be attributed to *T. thioparus* growth suppression function.

Key words: Carbon disulfide, bio-desulfurization, inhibition, *Thiobacillus thioparus*, resting cell, gases purification.

INTRODUCTION

Hydrogen sulfide (H_2S) is a highly toxic, corroded and malodor gas, which is a common ingredient in natural gas and biogas (Zhang et al., 2008; Kim et al., 2005). It was reported that the concentration of H_2S could get to as high as 17 000 ppm, when sulfate-rich wastewater was converted to biogas by anaerobic digestion (Chaiprapat

et al., 2011). Biological desulfurization is a novel method for the removal of H_2S from gases stream by sulfide oxidizing bacteria (SOBs) that have capacity of oxidizing low state sulfur compounds. In the process of biological desulfurization, H_2S is firstly adsorbed by alkaline adsorbent, and then is oxidized to element sulfur by SOBs under oxygen limitation (Equations 1 to 2). Profiting from the regeneration of hydroxide, the alkaline adsorbents could be recycled (Equation 2). It has been considered as the best alternative of the classic chemical methods for desulfurization, and has perspective application in the fields of the desulfurization of gases, such as natural gas and biogas (Abatzoglou and Boivin, 2009).

$$H_2S + OH^- \rightarrow HS^- + H_2O \quad (1)$$

$$2HS^- + O_2 \xrightarrow{SOBs} 2S + 2OH^- \quad (2)$$

In the raw natural gas or biogas, there are also some volatile organic sulfur compounds (VOSCs), such as methanethiol, dimethyl disulfide, dimethyl sulfide, carbon disulfide and carbonyl sulfide (Mata-Alvarez and Llabrés, 2000; Böresson, 2001; Sheng et al., 2008). These VOSCs could also be absorbed by the alkaline adsorbents, and make significant effect on the activity of SOBs (Lobo et al., 1999). It was reported that *Thiobacillus thioparus* DW44 had the closed specific uptake rates ($g \cdot S \cdot Cell^{-1} \cdot h^{-1}$) of H_2S (7.49×10^{-14}), methanethiol (3.45×10^{-14}), dimethyl disulfide (1.24×10^{-14}) and dimethyl sulfide (4.14×10^{-15}) (Kyeoung et al., 1991).

However, it had been reported that most of *Thiobacilli* were not only incapable of oxidizing carbon disulfide, but were inhibited by VOSCs, including *T. thiooxidans* ATCC 19377, *T. ferrooxidans* ATCC 23270, *T. neapolitanus* DSM 581, *T. versutus* DSM 582, *T. thioparus* DSM 505, *T. acidophilus* DSM 700, *T. thiooxidans* ATCC 8085, *T. aquaesulis* DSM 4255 and *T. tepidarius* DSM 3134 (Neil et al., 1988). Seldom acidic *Thiobacillus* strains were able to use carbon disulfide as sole energy source. For example, *Thiobacillus* strain TJ330 DSM 8985 that is similar to *T. thiooxidans* ATCC 19377 and *T. ferrooxidans* ATCC 23270 could take carbon disulfide as one of the substrates for growth (Hartikainen et al., 2000). Few literatures have reported the inhibitory mechanism of carbon disulfide on the biological desulfurization process.

One approach to enhance biological desulfurization focused on the carbon disulfide inhibition. Although the carbon disulfide slightly dissolved in the water, little carbon disulfide could obviously inhibit the growth of cells. In this paper, the effect of carbon disulfide on the biological desulfurization was investigated, and the inhibitory kinetics was proposed for the first time.

MATERIALS AND METHODS

Microorganism

In this study, *T. thioparus* CGMCC 4826 isolated from the effluent of sulfate reducing bioreactor in our laboratory was used. It had been proved that this strain was not able to grow on CS_2. This strain could oxidize thiosulfate to elemental sulfur and sulfate (Equation 3). Compared with sulfide, thiosulfate was not sensitive to chemical oxidation and non-toxic to cells. These benefits would make it easy to estimate the effect of carbon disulfide on desulfurization.

$$S\text{-}SO_3^{2-} + 1/2O_2 \xrightarrow{SOBs} S + SO_4^{2-} \quad (3)$$

Medium and Culture

The culture medium contained (g/L): KNO_3, 0.50; K_2HPO_4, 4.00; KH_2PO_4, 4.00, $MgSO4 \cdot 7H_2O$ 0.10, $CaCl_2$, 0.10; $FeCl_3 \cdot 6H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.02; $Na_2S_2O_3 \cdot 5H_2O$, 15.00 g. The pH was adjusted to 7.0 with 1.0 M NaOH or 1.0 M HCl. *T. thioparus* was pre-cultured at 30°C and180 rpm for 36 h. And then, it was inoculated in medium volume of 1.0 L in a 3.0 L bioreactor (Bioflo 110 fermenter, New Brunswick Scientific, Edison, NJ) at the ratio of 5% (v/v). With the pH value being controlled at 6.8 to 7.2, the concentration of dissolved oxygen was maintained at 4 to 6 mg/L for the adequate supply of oxygen.

Biomass assay

The biomass concentration was measured by the amount of total N and N consumption (van den Bosch et al., 2006). Thiosulfate was oxidized to elemental sulfur and sulfate by *T. thioparus*, so there were many sulfur granules suspended in the medium. Approximately, 90% of biomass was absorbed by the sulfur particles. As a result, it was difficult to accurately measure the concentration of cell by the method of turbidimetry and protein assay. On basis of the mass balance, the growth rate of cell was determined by the consumption rate of nitrate (Visser et al., 1997).

Desulfurization by resting cell

The resting cells were prepared by the method of N source limitation. The bacteria could not grow without N source. *T. thioparus* was pre-cultured at 30°C and 180 rpm for 36 h. The culture was centrifuged at 500 rpm for 10 min to remove elemental sulfur particles. The supernatant was centrifuged at 6 000 rpm for 10 min. The precipitation was washed twice by physiological saline (0.9% NaCl), and was resuspended by the medium without potassium nitrate and thiosulfate. After stirred at 30°C, 180 rpm for 30 min, the thiosulfate solution (10×) was added. If carbon disulfide was needed, it was added into medium before being stirred.

Analytical method

Samples were filtered over 0.22 μm membrane. Sulfate, thiosulfate

*Corresponding author. E-mail: jmxing@home.ipe.ac.cn. Tel: +86 010 62550913. Fax: +86 010 62550913.

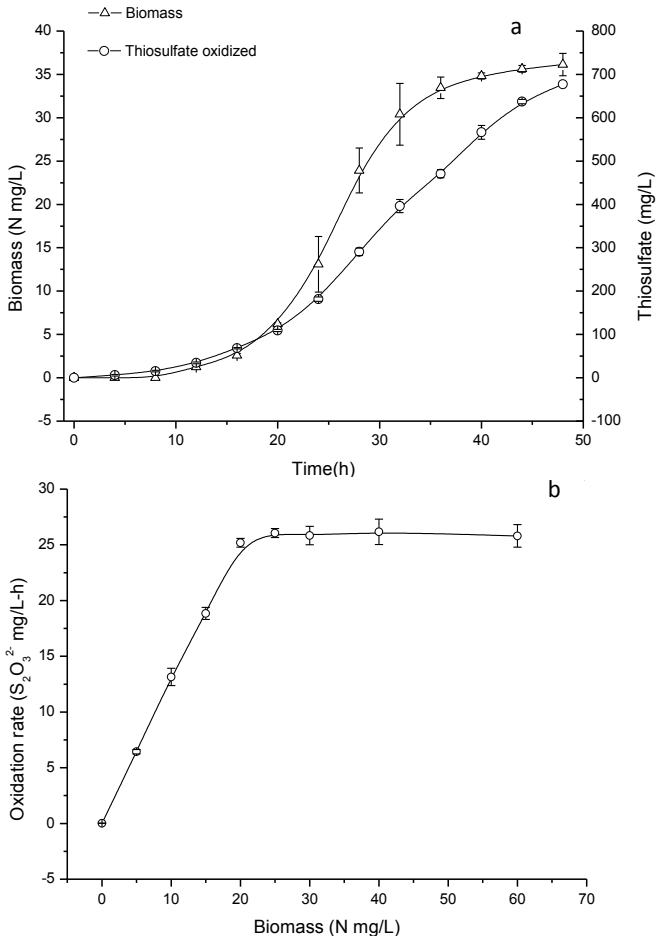

Figure 1. (a) The relation between cell growth and thiosulfate oxidation without carbon disulfide. **(b)** The desulfurization activity of the resting cell of *T. thioparus* under different cell density. The resting cell could be regarded as enzyme. The optimal density of resting cell was 20 to 25 N mg/L, and the corresponding maximum oxidation rate was about 25 $S_2O_3^{2-}$ mg/L-h.

and nitrate were analyzed by ion chromatography (Dionex model ICS 900, Dionex, Sunnyvale, CA), which was equipped with an electrical conductivity detector (Dionex Sunnyvale, CA). An ionpac AS14 column (Dionex Sunnyvale, CA) was operated at 25°C; the mobile phase was 8.0 mM Na_2CO_3/1.0 mM $NaHCO_3$ at a flow rate of 1.0 ml/min. The injection volume was 10 µL. The desulfurization activity of resting cells was determined by the method of termination reaction. After 60 min, the samples were taken from the reaction system, and immediately centrifuged at 10 000 rpm for 10 min to terminate the reaction. The samples were immediately analyzed by ion chromatography method. The unit of desulfurization activity was $S_2O_3^{2-}$ mg/L-h. The cells were cracked by sodium dodecyl sulfate (SDS) lysis buffer (Catalogue Number: 20 to 163, Millipore, USA), and the protein was stained with coomassie brilliant blue G-250 and the absorption value was measured at 595 nm (Hu et al., 2009). The average percentage of N in protein was 16% and the cell density was calculated by the below equation:

Cell density (mg-N/L) = 16% Protein content (mg-protein/L)

RESULTS

The growth of cells without carbon disulfide

As a control, *T. thioparus* was firstly cultured without carbon disulfide. The cell growth rate and the thiosulfate oxidation rate were investigated (Figure 1a). The delay phase of the cell growth was 20 h, and the platform phase appeared at the 40 h. The maximum of cell density was 36.13 mg-N/L. During the logarithmic phase, the average rate of the cell growth was 1.70 mg-N/L-h. It was found that the thiosulfate oxidation rate was not synchronously increased with the cell growth. After 28 h, there was a clear acceleration in the rate of thiosulfate oxidation. It was 12 h later than the logarithmic phase of the cell growth. When the concentration of cell was above 23.92 mg-N/L, the average rate of thiosulfate oxidation achieved the highest value, 26.50 $S_2O_3^{2-}$ mg/L-h. After the growth of cell had stopped, the thiosulfate oxidation rate was still kept at the constant level. These results suggest that the oxidation of thiosulfate was not completely attributed to the cell growth. The cells of *T. thioparus* mainly played a role of enzyme in the process of sulfur compounds oxidation, so that *T. thioparus* could be immobilized in the same way of enzyme (Qiu et al., 2006).

Effect of various concentrations of carbon disulfide on the cell growth

During the adsorption of hydrogen sulfide, carbon disulfide is absorbed into the absorbent. Carbon disulfide slightly dissolves in water, and its solubility in water is 0.20% at standard conditions. So, the maximum concentration of carbon disulfide in this experiment was set as 0.20%. To determine the effect of the carbon disulfide on the growth of *T. thioparus*, 0.01, 0.05, 0.10, 0.15, and 0.20% was added respectively to the medium with the other parameters unchanged. The results obviously showed that carbon disulfide had substantially negative effect on the growth of *T. thioparus* (Figure 2a).

When the concentration was 0.01%, the carbon disulfide extended the delay phase from 20 to 24 h. After that, *T. thioparus* was adapted to the carbon disulfide and presented similar curve of logarithmic growth to the control. The maximum cell density and the average growth rate of logarithmic phase were 35.62 and 1.59 mg-N/L-h, respectively (Figure 2b) both of them were closed to the bank control. However, under the condition of 0.05% carbon disulfide, the growth of cell was significantly inhibited by carbon disulfide. The maximum cell density and the average growth rate of logarithmic phase respectively decreased to 19.75 and 1.04 mg-N/L-h, which were only the 54.67 and 61.18% of the control group. The delay phase reached almost 32 h later. As the concentration of carbon disulfide increased to 0.10 and

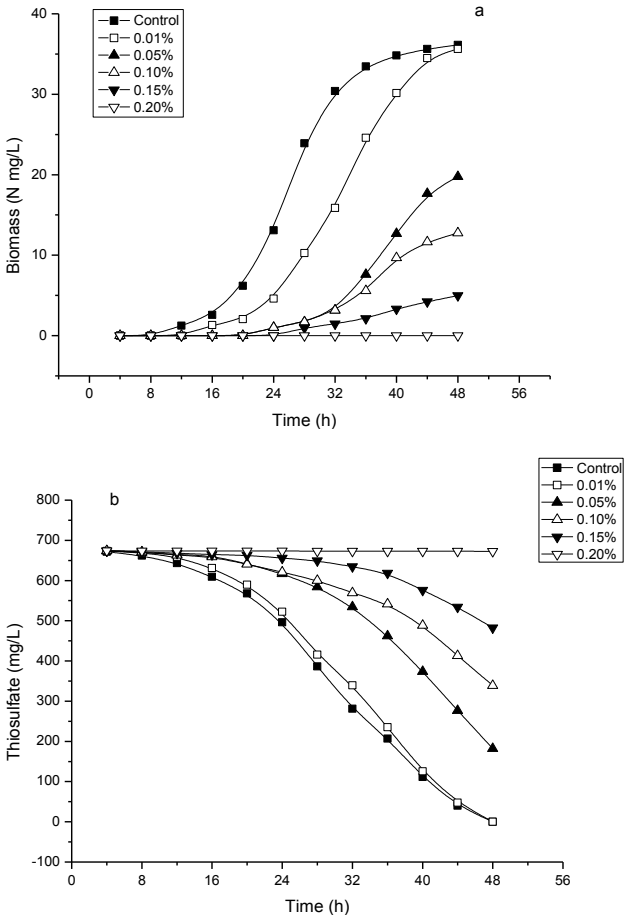

Figure 2. The effects of carbon disulfide on the thiosulfate oxidation and cell proliferation during cell growth. Various concentrations of carbon disulfide were added into the medium at the beginning of culture. It was obvious that carbon disulfide could negatively affect the growth of *T. thioparus* at the low concentration (a). However, there were no significant differences between control and 0.01% in the thiosulfate oxidized by the resting cell (b). It demonstrated that the thiosulfate oxidation was not coupled with the cell growth.

0.15%, the logarithmic phases of growth disappeared, the curves of growth cell were similar to the line. When the concentration of carbon disulfide reached 0.20%, the growth of cell was completely inhibited. The 0.05% carbon disulfide outstandingly inhibited the growth of cell, but the line of thiosulfate oxidation mostly paralleled the one of the control group. It suggested that carbon disulfide firstly affected the growth of cell, and then declined the desulfurization activity of cell.

Desulfurization activity of resting cell

On basis of the above results, it was deducted that the cell of *T. thioparus* could catalyze the thiosulfate oxidation in similar way as the enzyme. In this experiment, the growth of cell was limited by the absence of N resource, and the resting cell was taken as enzyme and the enzyme activity was estimated. In the middle of logarithmic phase, the cell was gathered by centrifu-gation, washed twice with physiological saline, and then resuspended by the medium without N resource. The cell density was measured by protein assay, and then was adjusted to 5, 10, 15, 20, 25, 30, 40 and 60 mg-N/L, respectively. The initial rate of thiosulfate oxidation was determined (Figure 1b). The initial rate reached the maximum value (25.19 $S_2O_3^{2-}$ mg/L-h) at the cell density of 20 mg-N/L.

Under the density of resting cell was set the optimal 20 mg-N/L, the concentration of substrate thiosulfate was changed to 0.5, 1, 2, 5, 10 and 20 g/L, and the initial rate of desulfurization was determined. The Michaelis-Menten equation was used for the process modeling. The K_m and V_{max} of the resting cell were calculated by the Lineweaver-Burk method (Kim et al., 2004; Li et al., 2008). The K_m and V_{max} were 0.264 mM and 27.93 $S_2O_3^{2-}$ mg/L-h, respectively (Figure 3a).

Inhibition of resting cell by carbon disulfide

In order to investigate the effect of carbon disulfide on resting cell, 0.05, 0.01 and 0.20% carbon disulfide was added to the solutions containing 10, 20, 30, 40, 50 mg-N/L resting cell. The initial rate of thiosulfate oxidation was detected after 60 min. In comparison with the control group, 0.05% carbon disulfide hardly impacted the rate of thiosulfate oxidation (Figure 4). When the concentration of carbon disulfide got to 0.10%, the rate of thiosulfate oxidation obviously decreased, but it could partially be restored with the increasing of biomass. In batch culture, the maximum cell density was about 40 mg-N/L. Under this cell density and 0.1% carbon disulfide, the rate of thiosulfate oxidation was only 14.0 $S_2O_3^{2-}$ mg/L-h (54.28% of the control group).

After that, the concentration of carbon disulfide was respectively set as 0.5 and 0.10%; the density of resting cell was 20 mg-N/L. The parameters of Michaelis-Menten equation was calculated by the method of Lineweaver-Burk (Figure 3b). When the concentration of carbon disulfide was 0.05%, the V_{max} was 26.77 mg $S_2O_3^{2-}$ /L-h, which was closed to the 27.93 $S_2O_3^{2-}$ mg/L-h of the control group. However, the K_m increased from 0.264 to 0.35 mM, and it continued to increase to 0.884 mM under 0.10% carbon disulfide.

DISCUSSION

In our experiments, the maximum cell density of *T. thioparus* CGMCC 4826 reached 36.13 mg-N/L, and the maximum growth rate was 10.9 mg-N/L-h. It had been reported that the *haloalkaliphilic Thioalkalivibiro* and *Thioalkalimicrbium* consortium used for hydrogen sulfide

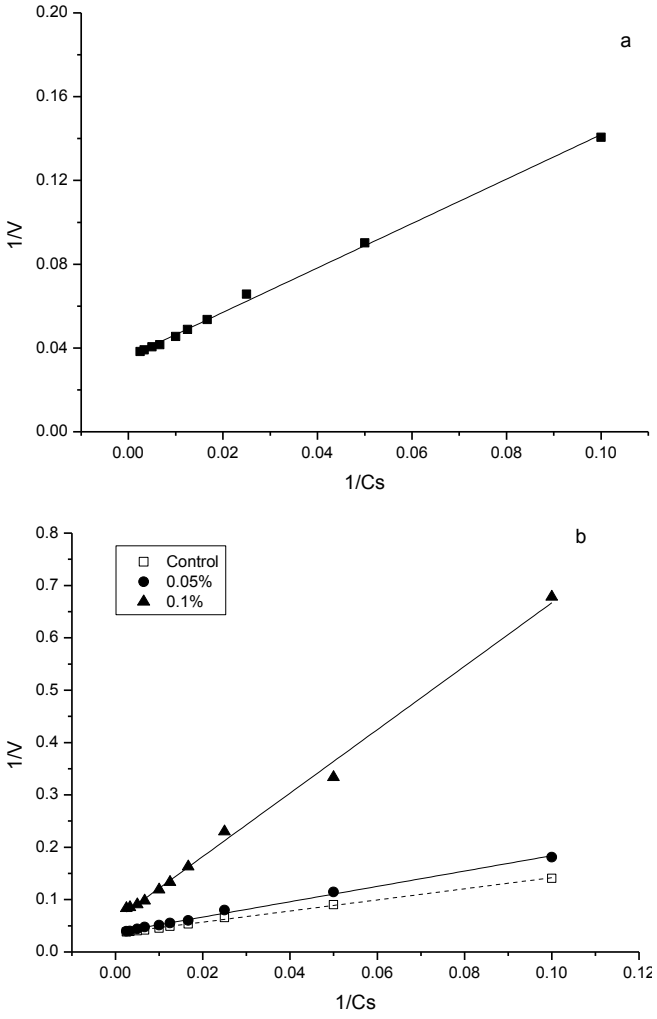

Figure 3. (a) The Michaelis-Menten parameters calculated by the method of Linewaver-Burk. K_m=29.61 mM, V_{max}= 27.93 $S_2O_3^{2-}$ mg/L-h (R^2=0.9979). **(b)** The effect of carbon disulfide on the Michaelis-Menten parameters of resting cell. When the concentration of carbon disulfide was 0.05%, the K_m and V_{max} were respectively, 39.21 mM and 26.77 $S_2O_3^{2-}$ mg /L-h. When it was 0.10%, the K_m and V_{max} were, respectively, 99.07 mM and 16.35 $S_2O_3^{2-}$ mg /L-h.

oxidation had the maximum growth rate of 7.8 mg-N/L-h under sulfate producing condition (van Den et al., 2009). The biomass concentration of *T. denitrificans* could increase from 350 to 600 mg-protein/L (56 to 96 mg-N/L) with 4.7 mM (65.8 mg-N/L) NH_4^+ utilized in batch cultivation, and it was about 180 mg-protein/L (28.8 mg-N/L) in a continuous stirred-tank reactor under aerobic conditions (Sublette, 1987). It could be deduced that *T. thioparus* CGMCC 4826 could grow very well on the thiosulfate medium with nitrate as N source under the strict aerobic condition.

According to the results, biomass growth rate was not consistent with the thiosulfate oxidation rate, which indicated that thiosulfate oxidation was not completely

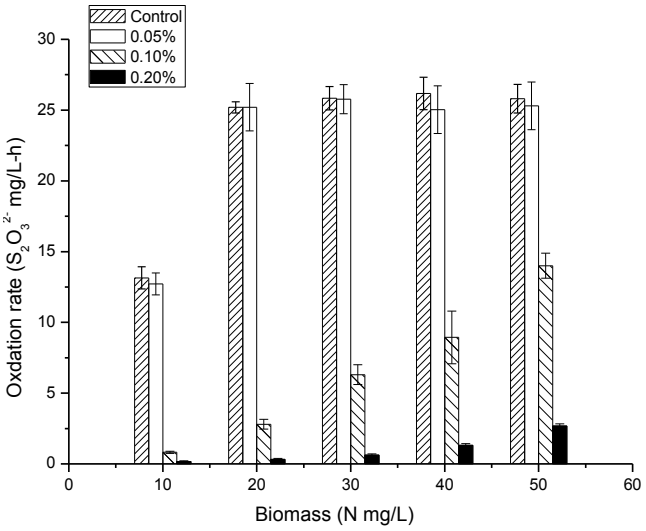

Figure 4. The effect of carbon disulfide on the desulfurization activity of resting cell. The 0.05% carbon disulfide hardly had effect on desulfurization activity of the resting cell.

coupled with biomass growth. Under the lower concentrations, the carbon disulfide violently inhibited the cell growth, but the thiosulfate oxidation was still running very well. The cells action in desulfurization was more like that of an enzyme.

This presumption could be proved by the immobilized SOBs, which were good for the enhancement of microbiological desulfurization. *Thiobacillus* sp. had been successfully immobilized into different material like Ca-alginate, K-carrageenan, agar and polyurethane by entrapment methods. After being pre-cultured 4 to 5 days, the immobilized *Thiobacillus* sp. could be reused for 10 cycles with the sulfide oxidation of 94 to 96% (Ravichandra et al., 2009). Thus, the resting cell of *T. thioparus* can also be investigated for the activity of desulfurization and the effect of carbon disulfide. The thiosulfate oxidation rate was rose up with the increase of biomass concentration, and its maximum was gained at the cell density of 20 mg-N/L.

Carbon disulfide was common ingredient in sour natural gas and biogas, some tail gases of plants. The concentrations of carbon disulfide in landfill gas from four landfill sites were in the range of from 25 to 5352 ppb, and the highest ratio of H_2S to CS_2 was about 6:1 (Kim et al., 2005), which was highly toxic to the strain *T. thioparus*.

The bad ability of removing carbon disulfide must lead to the accumulation of carbon disulfide in the recycle of adsorbents, which would negatively affect the biological desulfurization. When its concentration was 0.01%, *Thiobacillus* sp. could oxidize the carbon disulfide at pH 7.0 and 30°C, but the degradation activity was caused above levels of 0.015% (Plas et al., 1993). In this study, when its concentration was as low as 0.01%, carbon

disulfide could obviously decrease the cell growth rate. As the level was increased to 0.05%, the maximum of biomass was only 19.75 mg-N/L; 54.68% of the control group.

In contrast, carbon disulfide had less effect on the desulfurization activity of the resting cell. As its concentration was up to 0.05%, there were no obvious differences to the control batches. Under 0.05 and 0.1% carbon sulfide, the K_m of resting cell increased to 0.35 and 0.884 mM, respectively, which indicated that the affinity of resting cell to the substrate might decline. The thiosulfate was oxidized by the enzymes of Sox pathway located in bacterial periplasm, including a series of reactions (Bamford et al., 2002; Kelly et al., 1997). The possible reason was that carbon disulfides can affect the selective permeability of cell membrane, which leads to losing the ion balance of cell membrane. The $S_2O_3^{2-}$ or electron could not be correctly transferred to the sits of oxidase on the membrane.

Conclusion

The sulfur compounds oxidation was not coupled with the growth of *T. thioparus,* the cells could be considered as enzyme, and the optimal density of resting cell was 20 mg-N/L. Although the carbon disulfide slightly dissolved in the water, little carbon disulfide could obviously inhibit the growth of cells. Moreover, it would significantly inhibit the desulfurization activity of resting cell for its cell toxicity, when its concentration was above 0.15%. In the process of biological desulfurization, carbon disulfide could not be oxidized by the SOBs; otherwise it may accumulate to a higher amount in the adsorbent. It would cause significant inhibition of desulfurization by restraining the cells density to an optimal value. If its concentration was above 0.05%, it would directly inhibit the desulfurization activity of cells. Therefore, 0.05% was the dangerous value of carbon disulfide for the biological desulfurization. Thus, under the optimized bio-desulfurization process, it was necessary to control the concentration of carbon disulfide in the absorbent below 0.05% by renewing the absorbent.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Kinetics of anaerobic digestion of labaneh whey in a batch reactor

Ibrahim M. Abu-Reesh

Chemical Engineering Department, Qatar University, P.O. Box 2713, Doha, Qatar.

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In this work, anaerobic digestion of labanah whey was carried out in a 100 L batch reactor (RE-BIOMAS) at temperature of 30-40°C and pH 6 - 7. During the experiments, the biogas production and chemical oxygen demand (COD) concentration were recorded with time. During fermentation of labaneh whey, the pH drops dramatically due to the accumulation of volatile fatty acids that inhibits the activity of methanogens, resulting in a low gas yield and low methane content of the biogas. In a 28 days batch experiment at 36°C and pH 6.5, COD removal efficiency of 84% was achieved at initial COD of 18,000 mg/l. The cumulative biogas production was 20 L. Experimental data were fitted to the four kinetic models: Monod, Logistic, Contois and Tessier models. Comparison was made between model predictions and experiments for COD concentration. Tessier model gave marginally better fit than other models tested. Kinetic and stoichiometric coefficients were determined for the four kinetic models using Matlab nonlinear optimization function. Diluted labaneh whey (about 8000 mg/l COD) was treated to almost complete COD removal in a 10 days retention time and producing about 20 L of biogas. The COD removal was described well by first order kinetics with respect to substrate concentration. The modified Gompertz equation was used to describe the cumulative production of biogas with time. The equation kinetic constants were determined for labaneh whey and for diluted whey.

Key words: Labaneh whey, biogas, anaerobic digestion, methane production, dairy wastewater, modified Gompertz equation.

INTRODUCTION

"Labaneh" (also spelt labneh, labne or labni) is a popular type of fermented milk product in the eastern Mediterranean countries. Labaneh whey is the yellow-greenish aqueous portion of the milk that separates from the curds during labaneh manufacturing. Labaneh whey is a waste by-product of the dairy industry. It retains about 55% of the milk nutrients (Kumari et al., 2011). The disposal of labaneh whey is considered a major problem

E-mail: abureesh@qu.edu.qa. Tel: (+974) 4403 4136. Fax: (+974) 4403 4131.

Abbreviations: BOD, Biochemical oxygen demand; **COD,** chemical oxygen demand; **CSTR,** continuous stirred tank reactor; **HRT,** hydraulic retention time; **UAPB,** up-flow anaerobic packed bed; **UASB,** up-flow anaerobic sludge blanket; **VFA,** volatile fatty acid.

because of its high biological oxygen demand. Whey discharge into sewage treatment plants can cause serious problems to the plants and to the surface water. This concern has been heightened in recent years by the increased volume of whey and the more stringent legislative requirements for effluent quality. Approximately 85 kg of cheese whey is produced from 100 kg of milk (Kosikowski, 1977). Whey is composed of 93% water and 7% solid. The solids are composed of lactose, proteins, lipids and various salts. Labaneh whey contains about 5% lactose sugar which can be utilized as a carbon source by bacteria in biological treatment (aerobic and anaerobic). It is a major dairy waste in many Middle East countries. There is little information published on labaneh whey. Most of the information available in literature is about cheese whey. The chemical and physical properties of labaneh whey collected from the Jordan Dairy Company (Zarka, Jordan) were reported by Batshon (1980). It has a typical pH of 3.5, which is even lower than acid whey or sweet whey. In labaneh whey there is less lactose, more calcium, phosphorus, magnesium and lactic acid as compared to sweet or acid whey (Batarseh, 1995).

Biological treatment reduces the pollutants to a level that meet even the most stringent requirements. Aerobic digestion is very energy intensive and leads to a large amount of sludge as compared to anaerobic digestion in which most of the energy from whey is conserved in the produced biogas (about 60% methane) and little is used in formation of biomass. Some of the advantages of anaerobic digestion are the high energy efficiency, lower cost in addition to simplicity of the process as compared to aerobic processes.

Anaerobic digestion of labaneh whey offers an excellent solution in terms of both energy conversion and pollution control. Labaneh whey can be classified as acid whey (pH = 3.4) that makes pH control a serious problem during anaerobic digestion. No previous studies reported on anaerobic digestion of labaneh whey. All previous studies were on anaerobic digestion of cheese whey that has different compo-sitions and pH values. Anaerobic digestion of cheese whey in two stage configuration is more efficient than a single stage because of the possibility of optimization of operating conditions in both acidogenesis and metha-nogenisis processes. Jasko et al. (2011) showed that two phase bioreactor was a good choice for anaerobic digestion of cheese whey. In their experiment, they determined methane yield that fluctuated in the range of 136.6 - 216.3 l/kg volatile solid. Stamatelatou et al. (2012) used two stages consisting of a CSTR and UASBR to overcome inhibition during anaerobic treatment of diluted cheese whey. This system achieved 95% COD removal and produced 9 l/l d biogas. Also, Patil et al. (2012) used two stages up-flow anaerobic packed bed reactor for the treatment of cheese whey, they obtained 94-96% COD removal depending on the HRT.

Biogas production from cheese whey was enhanced by co-digestion of whey with other substrates. Various co-substrates have been reported in the literature such as sewage sludge (Powell et al., 2013), cow manure (Comino et al., 2009) and olive mill waste (Martinez-Garcia et al., 2007). Comino et al. (2012) studied biogas production and COD, BOD removal of a mixture of cattle slurry and cheese whey in a 128 L continuous anaerobic reactor. Using a mixture 50% slurry, biogas production was 621 l/kg VS at HRT of 42 days. The methane content of the biogas was 55%. The maximum percentage removal of COD and BOD were 82 and 90%, respectively.

Powell et al. (2013) studied the effect of cheese whey storage on the biogas production from co-digestion of whey and sewage sludge. Storage of whey affects its composition but has no effect on the methane production (m^3 CH_4/kgCOD_{added}). Najafpour et al. (2008) used successfully up-flow anaerobic sludge fixed film bioreactor to treat dairy wastewater and produce biogas. COD and lactose removal achieved were 97.5 and 98%, respectively, at HRT of 48 h. The highest production rate of biogas (3.75 l/day) was achieved at HRT of 36 h.

It is known that anaerobic digestion consists of three main steps: Hydrolysis of complex waste to monomers, oxidation of the products of the first step to acetic acids and CO_2 and the third step is formation of methane by methanogenic bacteria that use acetate, CO_2 and hydrogen to produce methane. Mathematical modeling of anaerobic digestion of labaneh whey is important for better understanding of the process. It is important for design, operation and prediction of the performance of the bioreactor that carry out the biological treatment process.

Yilmaz and Atalay (2003) developed mathematical model to describe substrate reduction and biogas production for batch reactor using five different organic wastes. The mathematical model predictions agree well with the experimental data. No mathematical model or kinetic study of anaerobic digestion is available in the literature for labaneh whey which has different compo-sition and pH than cheese whey. The objective of this work was to study the kinetics of substrate utilization and biogas production during batch anaerobic digestion of labaneh whey and diluted labaneh whey in a 100 L pilot plant.

MATERIALS AND METHODS

Characterization of labaneh whey

Labaneh whey used in this work was collected from dairy factory at the College of Agriculture, University of Jordan, Amman, Jordan. Labaneh whey was obtained at a pH slightly less than 3.5 with a green yellowish color. Collected whey was stored at 4°C before anaerobic digestion. Typical characteristics of labaneh whey are shown in Table 1 (Batshon, 1980).

Table 1. Chemical and physical properties of labaneh whey from Jordan Dairy Company (Batshon, 1980).

Lactic acid (w %)	Specific gravity (-)	Total solids (w %)	Total ash (w %)	Glucose (w/v %)	Lactose (w/v %)	Total nitrogen (w %)	Protein (w %)	Fat (w/v %)	pH
1.568	1.03	6.2	0.9	1.009	1.234	0.298	1.862	0.683	3.4

Figure 1. RE-BIOMAS-pilot plant for the production of biogas from biomass.

Anaerobic digestion pilot plant

The RE-BIOMAS (DIDACTA Italia) pilot plant was used in the anaerobic digestion study of labaneh whey (Figure 1). The 100 L reactor has a pH and temperature control system. Mixing was carried out using circulating pump at the bottom of the reactor. Variable power electric heater was used to heat the reactor. Several temperature probes were placed inside the reactor. Base solution is used to control the pH.

In the degassing settling tank, the gas is separated from the sludge that is recycled to the reactor. After this point, the gas is subjected to a series of treatment steps and the carbon dioxide is removed by absorption in water and basic solution. The gas is dried by silica gel filter. Finally, the hydrogen sulfide is removed by activated carbon bed. The gas is stored at atmospheric pressure in a volumetric tank. Liquid and gas samples were taken every day for COD and methane concentration measurements. The cumulative biogas volume was also recorded. Biomass used in this study was obtained from nearby domestic wastewater treatment plant (bottom of the thickener).

Analytical methods

COD was determined according to the standard procedure (APHA, 2005). The gas samples were collected in rubber balloon for analysis. Organic acids and evolved gases were analyzed by capillary gas chromatography-mass spectrometry (GC-MS) (PerkinElmer, AutoSystem XL Gas Chromatograph) according to Thaxton et al., 2010 (BrukerDaltonics Inc., MA 0182, USA) and ASTM D 1946-90 (2000), respectively. Lactose concentration was measured using phenol-sulfuric acid method (Dubois et al., 1965).

Modeling of batch anaerobic bioreactor

The kinetics of biomass growth can be determined by measuring either substrate (COD) consumption or product (biogas) formation with time. The second method is fast as compared to the first method. In this work, using growth kinetics and substrate balance around the batch reactor, a mathematical model was developed that describes COD reduction with time. The mathematical model predictions were compared with the experimental results for COD reduction and biogas production. The substrate balance around the batch bioreactor results is given as follows:

$$\frac{dS}{dt} = -Y_{xs}\,\mu\,X \quad S(t=0) = S_o = COD(o) \qquad (1)$$

Where, Y_{xs} is the yield coefficient (g/g), μ is the specific growth rate (d^{-1}), S_o is the initial substrate concentration (COD(o)), (g/l), and X is the cell (biomass) concentration (g/l). When the biomass yield coefficient is constant, the biomass concentration can be calculated using the following algebraic equation:

$$X = X_o + Y_{xs}(S_o - S) \qquad (2)$$

The following four biomass growth kinetic equations were used in this study.

Table 2. Variation of substrate concentration with time using four different kinetic models.

Kinetic equation	Differential equation	
Monod	$\frac{dS}{dt}=\left(\frac{-\mu_m S}{Y_{XS}}\right)\frac{\left[X_o+Y_{XS}(S_o-S)\right]}{K_S+S}$	(7)
Contois	$\frac{dS}{dt}=\left(\frac{-\mu_m S}{Y_{XS}}\right)\frac{\left[X_o+Y_{XS}(S_o-S)\right]}{k_{sx}\left[X_o+Y_{XS}(S_o-S)\right]+S}$	(8)
Logistic	$\frac{dS}{dt}=\left(\frac{-\mu_m}{Y_{xs}}\right)\left[X_o+Y_{XS}(S_o-S)-\frac{\left(X_o+Y_{XS}(S_o-S)\right)^2}{Y_{XS}S_o}\right]$	(9)
Tessier	$\frac{dS}{dt}=\left(\frac{-\mu_m}{Y_{xs}}\right)\left[X_o+Y_{XS}(S_o-S)\right]\left[1-e^{-\frac{S}{K_S}}\right]$	(10)

Monod equation $\mu=\mu_m\frac{S}{K_s+S}$ (3)

Contois equation $\mu=\mu_m\frac{S}{k_{sx}X+S}$ (4)

Logistic equation $\mu=\mu_m(1-\frac{X}{X_m})$ (5)

Tessier equation $\mu=\mu_m(1-e^{-\frac{S}{K_S}})$ (6)

Equation 1 that represents substrate concentration reduction with time is reduced to the following differential equations (Table 2) using the four kinetic models and assuming constant yield coefficients.

The above four kinetic models were fitted to the experimental data using the Matlab function (*fminsearch).*

First order kinetic model

In diluted labaneh whey experiments, the first order kinetic model was tested for COD reduction with time. Substrate balance around batch anaerobic digester assuming first order kinetics produces the following:

$$\frac{dS}{dt}=-k\,S \quad (S = S_o) \text{ at } t = 0 \qquad (11)$$

Where, k (d^{-1}) is the first order kinetic constant, k represents a measure of biodegradation rate. The higher the k value, the higher the biodegradability of the digester. Equation 11 was used to describe biodegradation of solid waste by anaerobic digestion (Chen and Hashimoto, 1979).

Integration of equation 11 yields:

$$\ln\frac{S}{S_o}=-k\,t \qquad (12)$$

Or in terms of COD $\ln\frac{COD(t)}{COD(o)}=-kt$ (13)

Or $\ln COD(t)-\ln COD(o)=-kt$ (14)

This is a straight line equation. When plotting ln COD(t) vs t, the slope will be - k and the intercept will be ln COD(o).

Kinetics of biogas production from labaneh whey

The modified Gompertz equation was used to describe the cumulative production of biogas. Equation (15) represents a modified Gompertz first order equation:

$$B_t=B\exp\left\{-\exp[\frac{R_b\times e}{B}(\lambda-t)+1]\right\} \qquad (15)$$

Where B_t is the cumulative biogas produced at any time (l); B is the biogas production potential (l); R_b is the maximum biogas production rate (l/d); λ is the lag phase (d), that is the time needed for the bacteria to acclimatize to the environment or the time needed to produce biogas.

The modified Gompertz equation is used by researchers to describe the cumulative biogas production during anaerobic digestion (Budiyono et al., 2010; Yusuf et al., 2011). This equation is based on the assumption that methanogens production rate of biogas in batch reactor corresponds to its specific growth rate.

Table 3. Chemical oxygen demand removal in the 28 days batch experiment.

Day	0	1	3	7	9	10	12	13	14	21	22	23	25	28
COD (g/l)	17.92	17.3	16.5	15.7	15	14.08	13.5	12.9	12.5	8	7	5.96	4	2.9
COD removal (%)	0	3.46	7.93	12.4	16.3	21.4	24.66	28	30.25	55.36	60.94	66.7	77.7	84

RESULTS AND DISCUSSION

Anaerobic digestion of labaneh whey was carried out in a 100 L pilot plant operating in batch at controlled temperature and pH. Liquid samples were collected for COD and VFA analysis. Gas samples were collected for measurements of methane and carbon dioxide concentrations. The biogas production was measured by liquid displacement system. Table 3 shows the COD reduction with time during the 28 days of the experiment at controlled temperature of 36°C and pH of 6.5. Four kinetic models were fitted to the experimental data using the Matlab function (*fminsearch)* that find the minimum of a scalar function of several variables (unconstrained nonlinear optimization) as shown in Figure 2. All the four models fitted the experimental data closely; however Teisser model marginally fitted the data better than other models tested (higher correlation coefficient). Table 4 shows the kinetic constants and error obtained from nonlinear curve fitting of the four kinetic models. Lactose in labaneh whey is converted easily to lactic acid and other volatile fatty acids by bacteria under anaerobic conditions.

This reduces the pH causing inhibition of the very sensitive methanogene bacteria. To keep the pH around 6, large amount of base was added to the reactor. The low pH caused reduction in the biogas production rate and low methane content of the biogas. Figure 3 shows the concentration of acetic and butyric acids in the reactor. The concentration of both organic acids increases with time and reached almost a steady state constant value. Anaerobic digestion studies of cheese whey at low pH showed lower productivity of biogas and low methane content of the produced biogas (Ghaly, 1996). In his experiments, he concluded that anaerobic digestion of cheese whey without pH control is not feasible.

Stamatelatou et al. (2012) used two stage systems to overcome this stability problem. In their work, they used a CSTR (2 l) followed BY UASB (6.3 l) reactor. This system produced 9 l/l d of biogas containing approximately 55% methane and achieved 95% COD removal.

Biogas production was measured with time until the change in production was very small. A total of 20 L of biogas was produced with 46% methane content. Figure 4 shows a third order polynomial fitting of the cumulative biogas volume vs. time as compared to experimental data. The modified Gompertz equation was also used to fit the cumulative biogas production (liter) with time (days). Figure 5 shows the experimental results and the modified Gompertz equation predictions. The equation kinetic constants B, R_b and λ were determined using nonlinear curve fitting of the Matlab function *lsqcurvefit*. The fitting correlation coefficient is close to 1. The biogas production potential B is 19.5 L while the maximum biogas production rate R_b is 6.5 l/d. The lag time λ is low (0.3 days = 7.2 h) as compared to batch experiments using other substrates (Yusuf et al., 2011).

Another anaerobic digestion experiment (Experiment 2) was carried out using dilute labaneh whey (initial COD of 8 g/l). Figure 6 shows the decline of COD with time. It is clear from this figure that almost complete COD removal was achieved in 10 days. During this experiment, the temperature and pH were controlled at 37°C and 6.5, respectively.

Plotting ln COD(o)/COD(t) vs. t yield a straight line equation with goodness of fit (correlation coefficient 0.9924) with slope (k) equal 0.24 day^{-1}. This figure shows that first order kinetics can be used to describe the COD removal for dilute labaneh whey (Figure 7). This is expected since other kinetic models such as Monod equation reduces to first order for low substrate concentration.

Using experimental data at three different temperatures (32, 35 and 37°C), the activation energy for anaerobic digestion of labaneh whey (E) can be determined. Plotting ln K vs 1/T(K) the activation energy can be calculated, assuming K vary with temperature according to Arrhenius equation ($k = k_o\, e^{-E/RT}$). As shown in Figure 8, k_o and E can be determined from the intercept and slope ($k_o = 6.146\ 10^{14}$) and the activation energy E = 21,850 cal/mole °K.

In the second type of experiments (anaerobic digestion of diluted labaneh whey), biogas production was measured with time until the change in production is very small. A total of about 20 L of biogas was produced in 10 days retention time. Figure 9 shows the experimental results and the modified Gompertz equation predictions. The goodness of fit is high (R = 0.965). The equation kinetic constants B, R_b and λ were determined, B = 20.15 L, while the maximum biogas production rate R_b is 3.35 L/day and the lag time λ is negative (λ = - 0.2648). No lag phase was observed during anaerobic digestion of diluted labaneh whey. The concentration of VFA during the 10 days experiment is shown in Figure 10. Acetic acid has higher concentration in this dilutted whey experiment as compared to that of undiluted whey experiment.

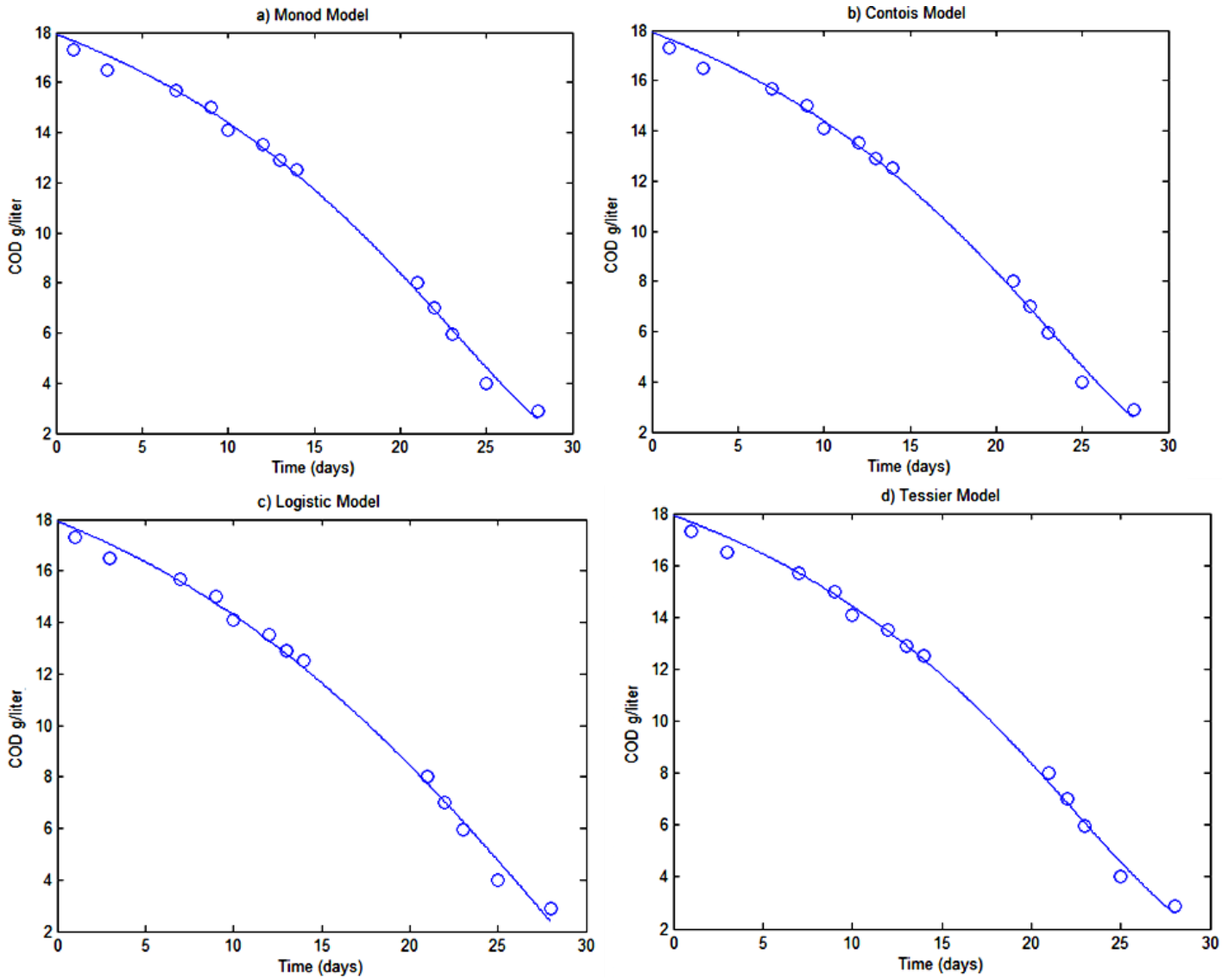

Figure 2. Biodegradation of COD with time using the 4 kinetic models (-o-o- experiment, _ model). a) Monod model; b) Contois model c) Logistic model; d) Tessier model.

Table 4. Kinetic constants and error obtained from non-linear curve fitting.

Kinetic equation	Kinetic constants	Smallest value of error
Monod	μ_m = 0.076 d^{-1}, k_s = 3.59 g/l	1.29
Contois	μ_m = 0.065 d^{-1}, k_{sx} = 1.27 g/l	1.29
Logistic	μ_m = 0.073 d^{-1}, X_m = 4.97 g/l	1.686
Tesseir	μ_m = 0.063 d^{-1}, k_s = 4.17 g/l	1.23

Conclusions

A 100 L pilot plant was used for anaerobic digestion and biogas production using labaneh whey as a substrate. Although the degradation of labaneh whey is very fast, accumulation of organic acids reduces the pH and caused instability in the system. This reduces the rate of biogas production and methane yield. The substrate

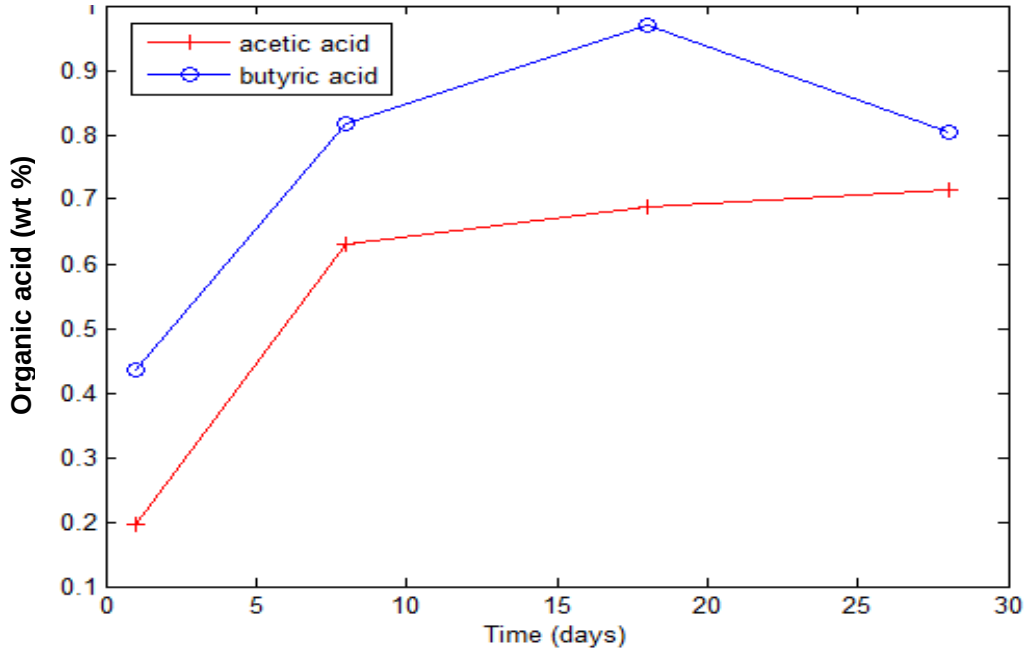

Figure 3. Concentration of acetic and butyric acid in the reactor.

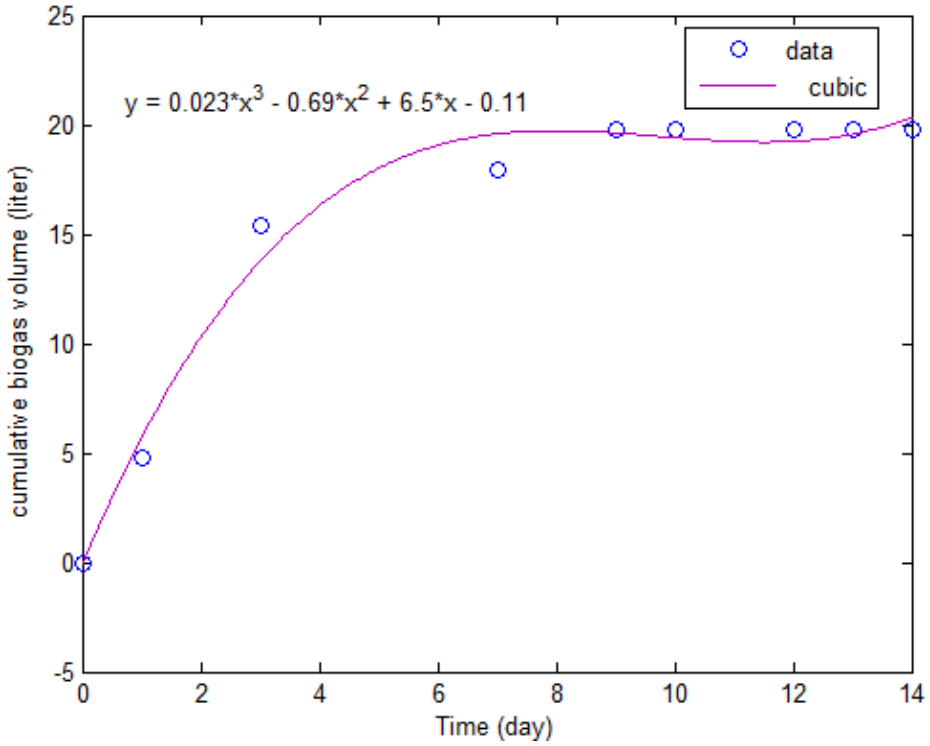

Figure 4. Cumulative biogas volume vs. time.

biodegradation was modeled and compared with the experiment. Four kinetic models were fitted closely to the experimental results of COD decline with time. Tesseir model gave marginally better fit than other models tested.

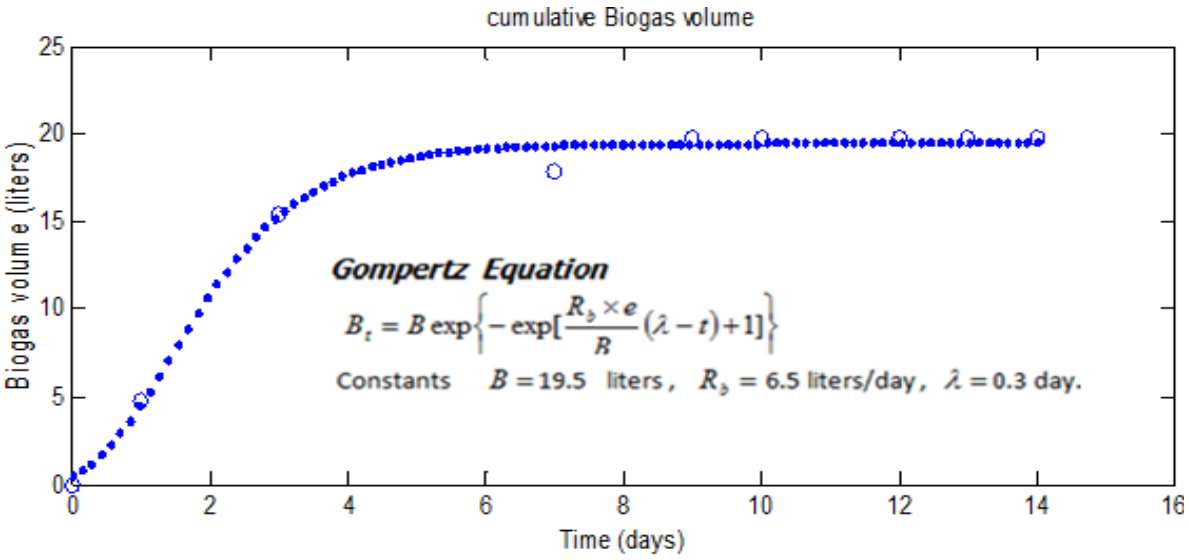

Figure 5. Kinetic constants and smallest value of error obtained from non-linear curve fitting of Gompertz equation (-o-o- experiment, ... model).

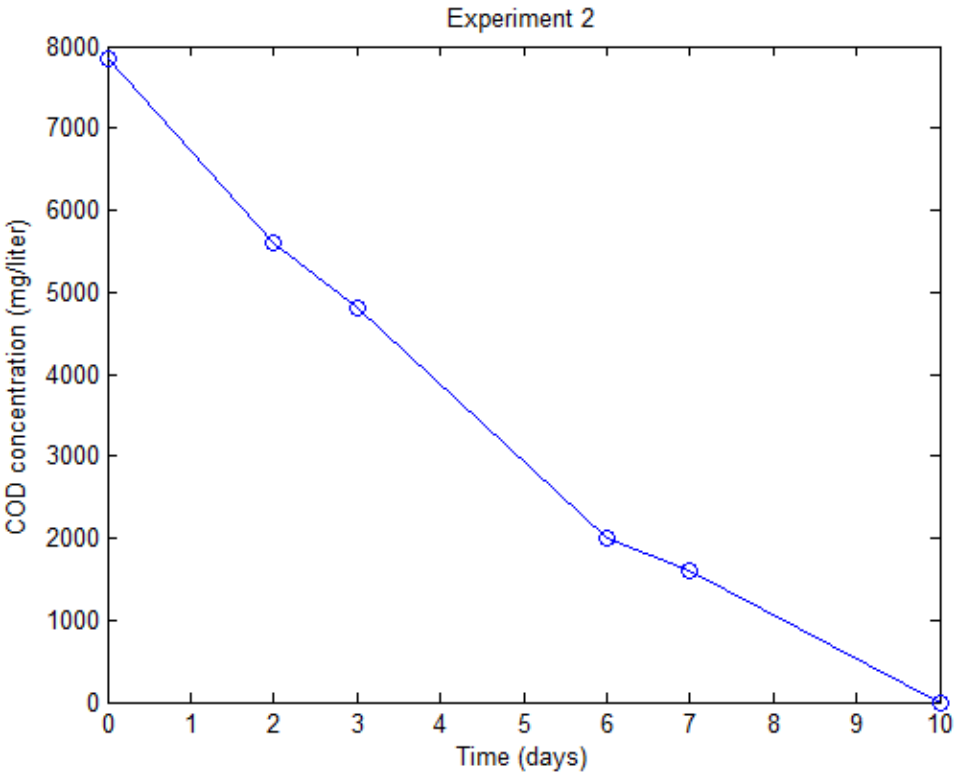

Figure 6. COD concentration vs. time during batch anaerobic digestion of diluted labaneh whey.

Percentage of COD removal achieved was 84%. The digestion process was unstable due to production of organic acids and rapid drop of pH that leads to low percentage of methane in the biogas (46%). 20 L of biogas were produced in a batch during 28 days of operation. Diluted labaneh whey was treated to almost

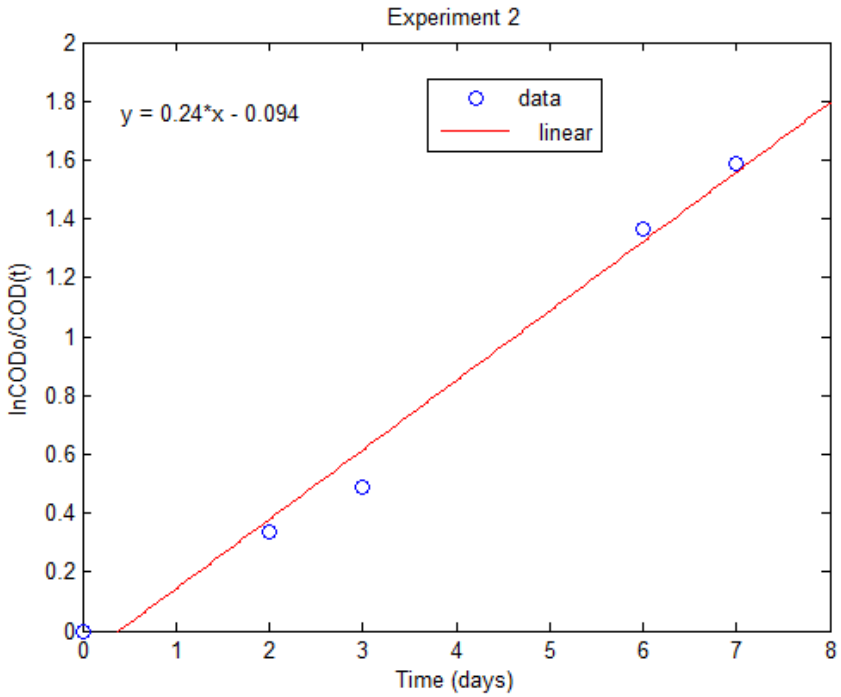

Figure 7. ln COD(t) vs. time (t) plot for anaerobic digestion of diluted labaneh whey (Experiment vs. model).

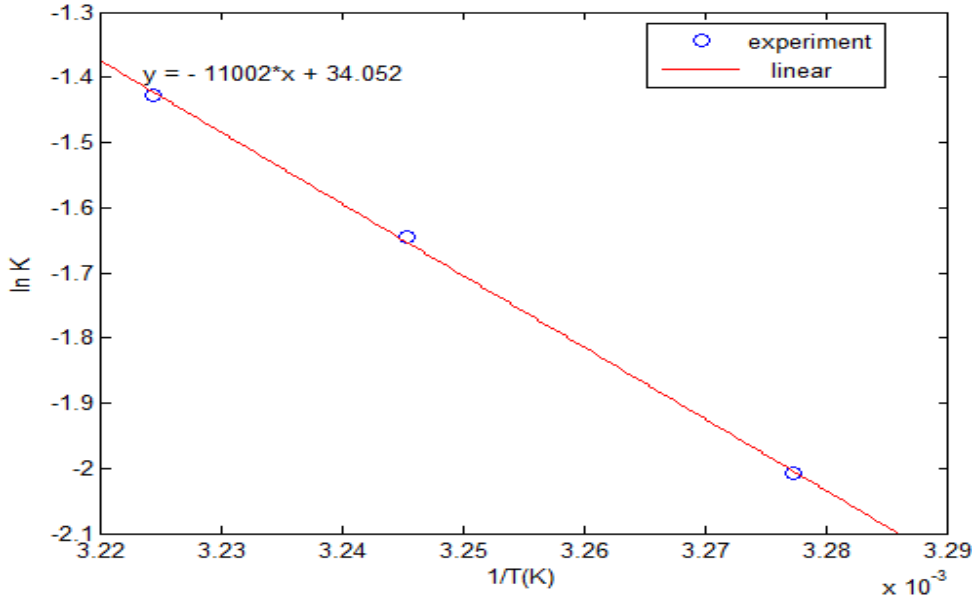

Figure 8. ln K vs. 1/T from anaerobic digestion experiments of diluted labaneh whey at three different temperatures.

complete COD removal in a shorter time and the COD removal was described well by first order kinetics. The modified Gompertz equation was used to describe the cumulative production of biogas with time. The equation kinetic constants were determined for both labaneh whey and for diluted labaneh whey.

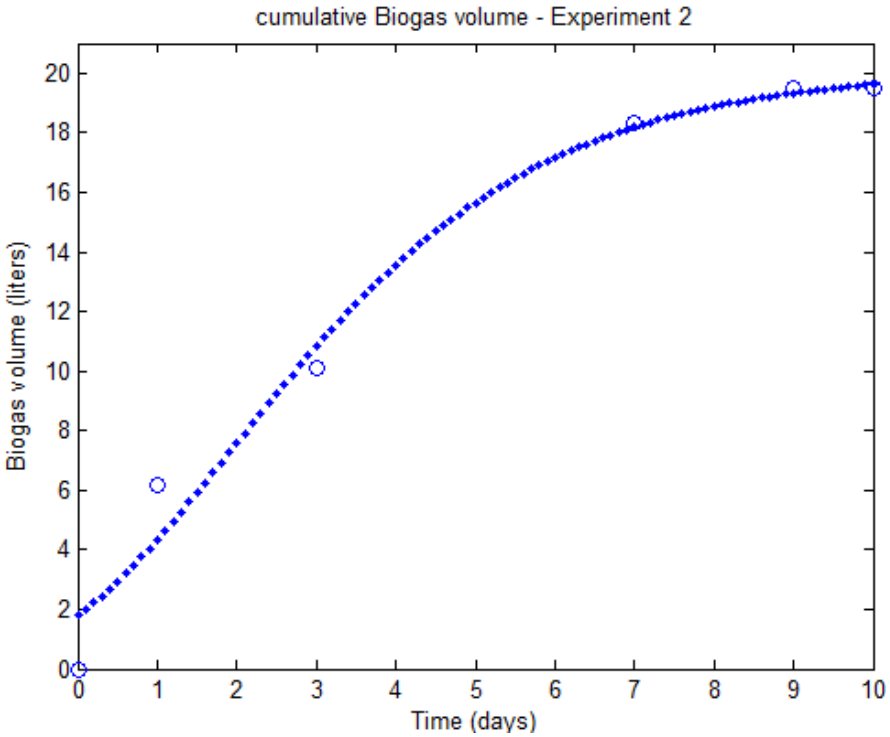

Figure 9. Kinetic constants and smallest value of error obtained from non-linear curve fitting of Gompertz equation (diluted whey) (-o-o- experiment, ... model).

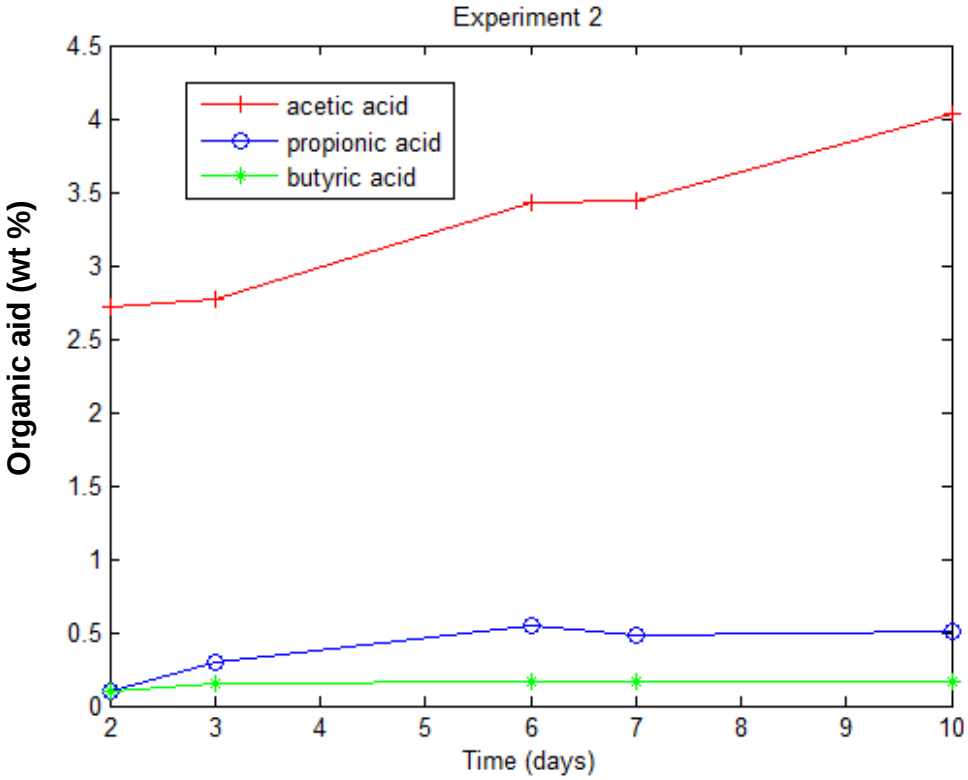

Figure 10. Concentration of volatile fatty acids in the reactor (diluted labaneh whey).

Conflict of Interests

The author(s) have not declared any conflict of interests.

Nomenclature

B, Biogas production potential (l)
B_t, Cumulative biogas production (l)
E, Activation energy in Arrhenius equation (cal/mole ^oK)
k, First order reaction rate constant (1/d)
k_o, Pre-exponential factor (1/d)
k_s, Constant in Monod, Tessier equations (g/l)
k_{sx}, Contois constant (g cell/g substrate)
R_b, Maximum biogas production rate (l/d)
S, Substrate concentration (g/l)
S_o, Initial substrate concentration (g/l)
t, Time (days)
T, Temperature (K)
X, Cell concentration (g/l)
X_o, Initial cell concentration (g/l)
X_m, Maximum cell concentration (g/l)
Y_{xs}, Cell yield coefficient (g cell/g substrate).

Greek letter

λ length of lag phase, d
μ specific growth rate, 1/d
μ_m maximum specific growth rate, 1/d

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