Production of fat and sterol by *Aspergillus nidulans* in submerged fermentation

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ABSTRACT

The efficiency of fat and sterol production by *Aspergillus nidulans* was evaluated. The fungus was grown in media containing various carbon and nitrogen sources. Some agricultural waste materials namely, corn cob, rice husk and potato peels were also used as sole carbon sources for fat and sterol production. Sodium acetate was added into culture media at various concentrations. After a 72 h incubation, mycelia obtained from culture media were assayed for fat and sterol contents. Among the sole carbon sources used for fat production, the highest yield of 48.63 mg/100 mL broth was achieved with mannitol after 72 h incubation. Carboxyl methyl cellulose was the poorest carbohydrate for fat production. The agricultural waste materials greatly improved fat production by *Aspergillus nidulans.* Levels of 44.62, 43.99 and 43.1 mg/100 mL broth were achieved with corn cob, rice husk and potato peels after 72 h incubation. Mannitol also caused the best sterol production by the fungus. Monosodium glutamate and corn steep liquor were the best nitrogen sources for the production of fat and caused the production of 64.36 and 64.27 mg/100 mL broth respectively. Urea and potassium nitrate did not support good fat production by the fungus. Hydrolyzed potato peels supported high yields of sterol as compared to mannitol. Increased fat production was accomplished through the addition of 0.1 to 0.5% sodium acetate. Maximum fat yield of 168.67 mg/100 mL broth occurred after 72 h in medium in which acetate concentration was 0.4%. This level was significantly higher than that of the control which had only 34.82 mg/100 mL broth of fat after 72 h. Acetate addition also caused reasonable increases in sterol synthesis higher than the control.

Key words : Fat; sterol; *Aspergillus nidulans*; submerged fermentation.

Introduction

Lipids have many metabolic roles, for example, they act as storage materials in animals, plants and microbial cells and are also responsible for the structure of cell membranes, and protect the body against cold and other environmental influences, etc. One of their important physiological roles is that they are precursors of hormone-like compounds, which is performed mainly by polyunsaturated fatty acids. It is essential to find new sources for lipids with concern to the nutritional problems accompanying the rapid growth of world's population. In fear of the depletion of oil resources and the global warming, present biotechnological research has concentrated on the commercial exploitation of microorganisms for the production of lipids. The possibility of cultivating microorganisms on extremely large scales for the production of single cell protein led to the realization that microorganisms can compete on equal terms with cheap plant products provided that the scale of operation is efficient.

A number of microorganisms belonging to the genera of yeast, fungi, bacteria and microalgae have ability to accumulate substantial amounts of oils, sometimes up to or even in excess of 70% of their biomass weight under specific cultivation conditions and many fungal species, such as *Aspergillus terreus*, *Claviceps purpurea, Tolyposporium, Mortierella alpina, Mortierella isabellina*, can accumulate lipids [1]. For nearly 100 years, the commercial opportunities of using microorganisms as sources of oils have been continuously examined. Although it was evident that microbial oils could never compete commercially with the major commodity plant oils,

there were commercial opportunities for the production of some of the higher valued oils. Today, with the great progress of metabolic and genetic engineering, the developments focus on the high value oils containing important polyunsaturated or specific fatty acids. Such oils have the potential to be used in different applications areas as food, feed and oleochemistry. A number of studies have shown that sterol and its peroxides might contribute to potential health benefits, including reducing pain related to inflammation, reducing the incident of cardiovascular disease, and inhibiting the tumor development [2, 3, 4] by direct inhibition of angiogenesis [3] and acting as an antioxidant and/or as anti-inflammatory agents [5]. Sterol molecules are essential for maintaining the proper structure and function of eukaryotic cell membranes [6].

Not all microorganisms can be considered as abundant sources of oils and fats. Like all living cells, microorganisms always contain lipids for the essential functioning of membranes and membranous structures. Organisms, principally eukaryotes, which can accumulate 20% or more of their biomass as lipid have been termed as "oleaginous" in keeping with oil-bearing plants that are similarly named. In fungi, lipids occur not only as major constituents of membrane systems, but also as cell wall components, as storage materials in abundant and readily observed lipid bodies and, in some cases, as extracellular products. The greater cell-size and complexity of fungi is accompanied by a corresponding diversity of lipid components. The amounts and types of lipid at individual fungal sites vary not only from one organism to another but also with age, stages of development, nutritional and environmental conditions. The lipid content of fungal species can be improved by varying culture conditions. Microbial lipids have been known

as a source of special oils and fats with high industrial potential for application and evaluated as an alternative source of animal and plant oils. They have the potential to be used for interesterification and as a substitute for certain dietary lipids. The production of sterols, more particularly ergosterol, by micro-organisms is now a well-established fact. It is, especially, a metabolic accomplishment characteristic of the moulds and yeasts. The objective of this present work is to develop methods for efficient production of fat and sterol from *Aspergillus nidulans* and to use some agricultural waste materials for lipid production.

MATERIALS AND METHODS

Isolation of fungus:

Soil samples were collected near a local palm oil producing factory into screw-capped conical flasks. The sample was serially diluted using normal saline solution. Diluted samples were plated out on Potato Dextrose agar (PDA) plates containing 0.1% chloramphenicol solution to inhibit bacterial contaminants. The plates were incubated for 48 h at room temperature (30±2^{o}C). Pure cultures of the isolates were obtained by streaking slant cultures on fresh PDA plates.

Preparation and collection of mycelium

The mycelium required for fat and sterol determinations was prepared by growing isolated culture, *Aspergillus nidulans* in shake flasks. Spores from 8-day old culture were added into 500 mL Erlenmeyer flask which contained 100 mL of medium which was designated as Medium A and had the following composition (in gram): glucose 2.5; $(NH_4)_2$ SO_4, 1.0; KH_2PO_4, 0.8; Na_2HPO_4, 0.5; $MgSO_4$.$7H_2O$, 0.2. The pH was adjusted to 5.8 after sterilization with sterile lactic acid and the flask incubated for 72 h on a rotary incubator shaker at 50 x g at 30^{o}C.

Carbon sources employed:

Fructose, sucrose, lactose, mannitol, maltose, carboxy methyl cellulose (CMC) and starch were individually employed as pure carbon sources. In a given series of fermentation flasks, each sugar was substituted for glucose as the carbon source in Medium A. When glucose was under test, it was retained in that series of fermentation flasks.

Corncob, potato peels, and rice husks were air dried, ground into powder using Corona mill (Medellin, Colombia) in 1L of deionized water. This was followed by dilute acid pretreatment (1% v/v H_2SO_4, 121^oC, 1 h). After pretreatment, the sample was filtered with a mesh sieve and re filtered with Whatman No 1 filter paper. All carbohydrates were each incorporated into media at 2.5% level and the pH adjusted to 5.8 using sterile lactic acid.

Into each fermentation flask were inoculated with 2 x 10^7 spores/mL of *Aspergillus nidulans* and added individual carbon sources under test. Flasks were allowed to shake at 50 x g for a maximum of 72 h, duplicate flasks harvested at 24 h intervals. The mycelia growth in each flask was collected through Whatman No 1 filter paper previously dried to constant weight. Mycelia were dried for 24 h in an oven at 100^oC and cooled prior to weighing on an analytical balance.

Effect of nitrogen substrates on lipid and sterol synthesis by *Aspergillus nidulans*: Into Medium A were added various nitrogen sources at 1% w/v concentration in place of ammonium sulphate. The medium pH was adjusted to 5.8. Inocula of 2 x 10^7 spores/mL of *Aspergillus nidulans* were added into each flask and incubated as described above.

Effect of acetate supplementation: Sodium acetate was added as supplement into Medium A which contained mannitol in place of glucose. Acetate was added at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5%. The flasks were each inoculated with 2 x 10^{7} spores/mL of *Aspergillus nidulans* and incubated as described above.

Determination of fat:

The procedure of Haas and Fleischman [7] was used. The mycelia obtained from the fermentation flasks were extracted with petroleum ether in a blender cup for 2½ min. The blender was operated at 12,000 rpm. The cup was immersed in a cold water bath during the extraction procedure. The extracted material was concentrated over a steam bath, dried at 105^{o}C for 75 minutes, cooled to room temperature in a dessicator charged with calcium chloride and weighed.

Determination of sterols: Sterols were extracted from the mycelia according to the procedure of Kieber *et al.* [8]. The mycelia were disrupted with 5% NaOH and sterol extraction carried out with ethyl ether. The extracted sterols were assayed by the Lieber Mann – Burchard reaction according to Stoudt and Foster [9]. Ergosterol was used for the construction of standard curve. Duplicate samples were included in each experiment. The data reported represent average values.

RESULTS AND DISCUSSION

Fat produced by *Aspergillus nidulans* occurred in the presence of all the carbon sources tested (Table 1). The highest yield of 48.63 mg/100 mL broth was achieved with mannitol after 72 h incubation. Lower yields of fat obtained with mannitol occurred at 24 h cultivation. Glucose sustained fat production throughout each time period. Fat concentration of 21.61 mg/100 mL broth was obtained after 24 h incubation. The most significant yield of fat in the presence of glucose occurred after 72 h. At this time, a yield of 34.88 mg/100 mL broth was obtained in flask signifying a 62% increase in yield. Fat yield of 33.96 mg/100 mL broth was obtained after 72 h with sucrose as the carbon source, however this level was not as high as those obtained with mannitol and glucose. CMC was the poorest carbon substrate for fat production. Fat yields of 2.22 mg/100 mL broth occurred after 24 h and increased poorly to 11.34 mg/100 mL broth after 72 h.

Microorganisms can grow and accumulate lipids on a wide range of carbon sources. The potential of an organism as an economic producer of lipid is related to the efficiency of substrate utilization. Carbon sources can strongly influence the production of fats by fungi due to differences in their metabolism; glucose, lactose, starches, oils, corn steep liquor, and agricultural produce have been used as carbon sources for production of lipids from fungi [10]. Other carbon sources such as xylose, arabinose,

mannose, mannitol and ethanol have also been investigated for the production of microbial lipids [11]. Glucose is the carbon source most commonly employed for growth of oleaginous fungi and lipid production [12, 13, 14]. To reduce the cost of microbial oils, exploring other carbon sources instead of glucose is very important especially for such oils applied to biodiesel production. It was reported that glycerol, corn straw, molasses, whey and other agricultural and industrial wastes could be used as carbon sources for microbial oil accumulation [15].

This finding shows that mannitol was most readily utilized by *Aspergillus nidulans* for the production of fat. Investigations with other microorganisms demonstrated that glucose was most suitable for the production of fat by various fungi [14]. Papanikolaou et al. [16] used *Mortierella isabellina* and *Cunninghamella echinulata* and showed the abilities of these organisms to accumulate lipids when grown on glucose as a carbon source. Burja *et al*. [17] used a strain that has a high biomass production rate, 25% higher than that exhibited by *Schizochytrium* sp. ATCC 20891; moreover this strain was able to accumulate up to 80% of its biomass as lipid when it was cultivated in a media containing glucose (60 g L^{-1}), yeast extract (2 g L^{-1}) and monosodium glutamate (8 g L^{-1}) with sea salts at 6 g L^{-1}. The biomass and lipid production by *Schizochytrium mangrovei*, was evaluated in a medium that contained glucose (40 g L^{-1}), yeast extract (20 g L^{-1}) [18]. Maximum lipid yields of 15.62, 14.48, 12.75, 13.68 and 20.41g L^{-1} were observed for *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* respectively [10]. *Schizochytrium* sp. F26-b, a strain isolated from Ishigaki island in Japan, produced 915 mg of lipids L^{-1} when it was inoculated into a medium containing glucose (3%), yeast extract

(1%) in 50% water, pH 6.0 [19]. The fractions of neutral lipids, glycolipids, and phospholipids corresponded to 66%, 7%, and 22%, respectively.

The use of corn cob, rice husk and hydrolyzed potato peels yielded maximum fat production of 44.62, 43.99 and 43.1 mg/100 mL broth respectively after 72 h. This level of fat is comparable to that produced with mannitol as the sole carbon source (Table 1). Many fungi have been screened, particularly for utilization of inexpensive industrial waste products like those from agricultural wastes for lipid production. Lignocellulose has attracted a lot of attention as feed stocks for bio fuel production due to its abundance and relatively low cost [20, 21]. Muniraj et al. [22] used potato processing waste water for microbial lipid production as a means of recycling potato processing waste water. The authors found the dilution ratio of 25% to be optimum for lipid production and the maximum lipid concentration obtained was 3.5g L^{-1}. The cellulolytic fungus, *Aspergillus oryzae* A-4, yielded a lipid content of 36.6 mg g^{-1} dry substrate by direct microbial conversion of wheat straw in suspended cultures and 62.87 mg g^{-1} dry substrate in solid substrate fermentation under optimized conditions [23]. Economou et al. [24] produced single cell oil from rice hulls hydrolysate while Venkata et al. [25] reported an efficient method for bio diesel production on corn cob using *Aspergillus* sp.

As was the case with fat production, the levels of sterol accumulated by *Aspergillus nidulans* varied with the carbon source employed. Although sterol was synthesized from each carbohydrate tested, mannitol allowed highest yields (Table 2). Highest level of sterol produced with this

carbohydrate was 6.88 mg/100 mL broth after 72 h. Sterol yields from CMC and starch were lowest than those obtained with other carbohydrates. Hydrolyzed potato peels supported high yields of sterol as compared to mannitol (Table 2).

This investigation was conducted to yield information on fat and sterol synthesis by *Aspergillus nidulans* under shaken cultivation. The first series of experiments illustrated the effect of different carbohydrates on fat and sterol production. This was followed by a study of the effects of different nitrogen sources on fat and sterol production. Monosodium glutamate and corn steep liquor were the best nitrogen sources for the production of fat and caused the production of 64.36 and 64.27 mg/100 mL broth respectively (Table 3). Urea and potassium nitrate did not support good fat production by the fungus.

All the tested nitrogen sources supported sterol production at various levels and the best yields occurred in media containing tryptone and yeast extract as sole nitrogen substrates (Table 4). Xue et al. [26] successfully grew the oleaginous yeast *Rhodotorula glutinis* with monosodium glutamate waste water to produce 25 g L^{-1} biomass with 25% of lipid content. Eroshin et al. [27] reported production of as much as 4.5 g L^{-1} of lipid by *Mortierella alpine* with a productivity of 19.2 mg L^{-1} h^{-1} with potassium nitrate as nitrogen source. Aki et al. [28] succeeded in producing 7.1 g L^{-1} of lipid using the fungus *Mortierella alliacea* in a 50-L jar with a 25-L working volume; a medium containing 12% glucose and 3% yeast extract produced 46.1 g L^{-1} cells with 42.3% lipids in 7 days. Enhanced biomass of 28.1 g L^{-1}

and a lipid content of 62.4% were achieved for *Trichosporon fermentans* by Zhu et al. [29] with peptone as nitrogen source, glucose as carbon source. Seven isolates of *Syzygites megalocarpus* were screened by Weete et al. [30] for their ability to produce lipids when cultured for 6 days in yeast extract/dextrose (YD) (2% glucose, 1% yeast extract) medium at 24°C. Biomass production in these isolates ranged from 2.9 to 9.7 g L^{-1} and total lipid content ranged from 4.0 to 9.8% of the dry mycelium

This work showed that mannitol caused the best fat and sterol production by *Aspergillus nidulans*. Therefore to test the effect of acetate supplementation on fat and sterol production, glucose in Medium A was replaced with mannitol. Increased fat production was accomplished through the addition of 0.1 to 0.5% sodium acetate (Fig. 1). Maximum fat yield of 168.67 mg/100 mL broth occurred after 72 h in medium in which acetate concentration was 0.4% (Fig. 1). This level was significantly higher than that of the control which had only 34.88 mg/100 mL broth of fat after 72 h.

The effects of acetate addition on sterol production is shown in Fig. 2. Although acetate caused very significant increases in fat production, only reasonable increases were observed for sterol synthesis. Addition of sodium acetate to medium at a level of 0.3 %, 28.65 mg/100 mL broth of sterol was produced after 72 h and this was higher than the control sample that had only a sterol concentration of 3.27 mg/100 mL broth after 72 h. Lehrian et al. [31] observed a 30% stimulation of mycelial growth of *Agaricus bisporus* upon the addition of small amounts of sodium acetate to complex media. The authors proposed that sodium acetate acted as a micronutrient

stimulating an enzyme or enzymatic pathway leading to the synthesis of lipid.

Conclusion

This study demonstrated that *Aspergillus nidulans* was suitable for studies dealing with microbial production of fat and sterol. The use of mannitol and agricultural waste materials namely, corn cob, rice husk and hydrolyzed potato peels as sole carbon sources in growth media caused highest fat and sterol production by the fungus. Best nitrogen substrates for fat production were monosodium glutamate and corn steep liquor. The presence of yeast extract and tryptone caused the best production of sterol by the fungus. Addition of sodium acetate in culture media greatly enhanced fat and sterol synthesis. The exploration of the natural biodiversity is a promising strategy to identify oleaginous microorganisms that produce fat and sterol on agro-industrial residues, particularly the lingo-cellulose materials. Cheap carbon sources have necessarily to be used as carbon sources for the cultivation of these organisms and the performance of the bioprocess has to be further improved in terms of yield and productivity.

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Table 1. Influence of carbon sources on the production of fat by *Aspergillus nidulans.*

Carbon source (2.5 %, w/v)	Period of incubation (h)		
	24	48	72
	Fat mg/100 mL broth)		
Glucose	21.6	29.91	34.88
Fructose	18.2	26.8	30.11
Lactose	10.83	21.16	31.63
Sucrose	21.43	28.3	33.94
Mannitol	21.91	33.01	48.63
Maltose	10.52	22.12	22.61
Carboxy methyl cellulose	2.22	5.86	11.34
Starch	6.62	11.59	21.82
Corn cob	21.85	33.55	44.62
Rice husk	26.68	33.16	43.99
Potato peel	21.47	32.35	43.1

Table 2**.** Influence of carbon sources on the production of sterols by *Aspergillus nidulans.*

Carbon source (2.5 %, w/v)	Period of incubation (h)		
	24	48	72
	Sterol (mg/100 mL broth)		
Glucose	1.12	2.31	3.27
Fructose	1.18	3.27	4.3
Lactose	2.09	3.16	4.14
Sucrose	3.81	4.56	6.33
Mannitol	3.3	5.29	6.88
Maltose	2.21	3.12	4.61
Carboxy methyl cellulose	0.06	0.12	0.61
Starch	1.1	2.13	3.18
Corn cob	2.26	4.59	4.66
Rice husk	3.21	4.23	5.21
Potato peel	2.16	5.38	6.81

Table 3. Influence of nitrogen sources on the production of fat by *Aspergillus nidulans.*

Nitrogen source (1 %, w/v)	Period of incubation (h)		
	24	48	72
	Fat mg/100 mL broth)		
Monosodium glutamate	24.82	46.35	64.36
Ammonium sulphate	21.61	29.91	34.88
Yeast extract	21.64	29.09	33.66
Peptone	26.36	31.66	43.98
Tryptone	14.58	28.85	34.01
Corn steep liquor	16.85	34.66	64.27
Urea	11.62	14.42	23.08
Potassium nitrate	5.59	9.25	10.74

Table 4. Influence of nitrogen sources on the production of sterols by *Aspergillus nidulans.*

Nitrogen source (1 %, w/v)	Period of incubation (h)		
	24	48	72
	Sterol (mg/100 mL broth)		
Monosodium glutamate	1.38	2.27	3.98
Ammonium sulphate	1.12	2.31	3.27
Yeast extract	1.86	3.18	4.64
Peptone	0.39	2.03	3.18
Tryptone	1.33	2.91	4.86
Corn steep liquor	1.46	2.35	3.47
Urea	0.18	0.26	0.34
Potassium nitrate	0.16	0.28	0.39

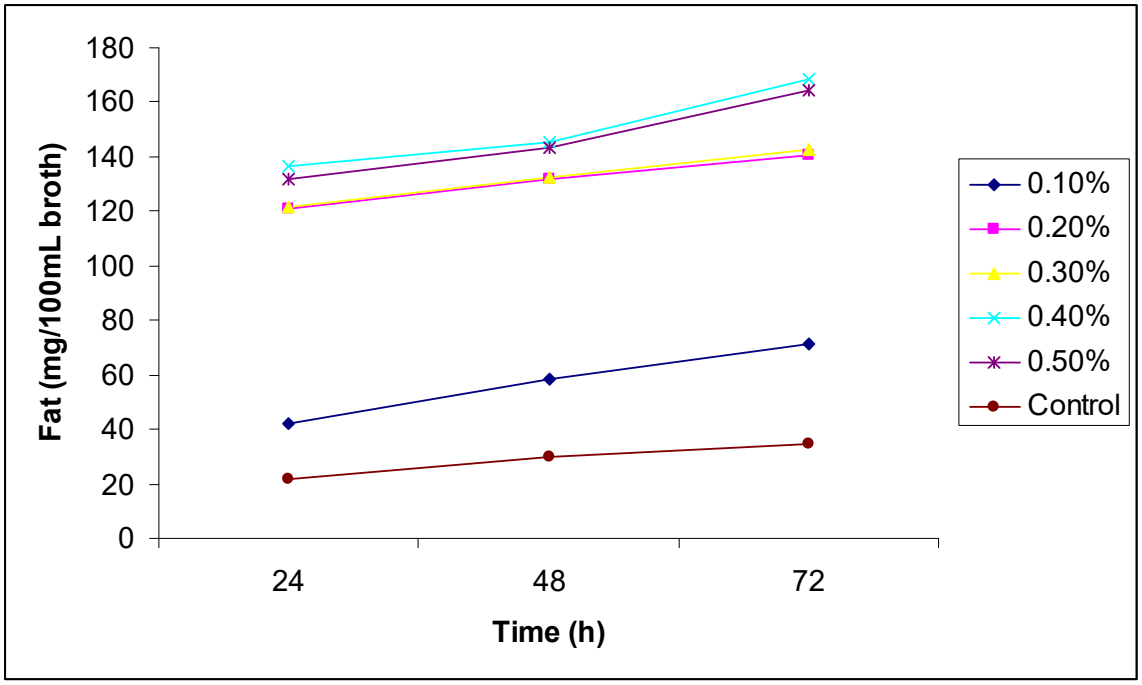

Fig.1. Effects of sodium acetate supplementation on fat production.

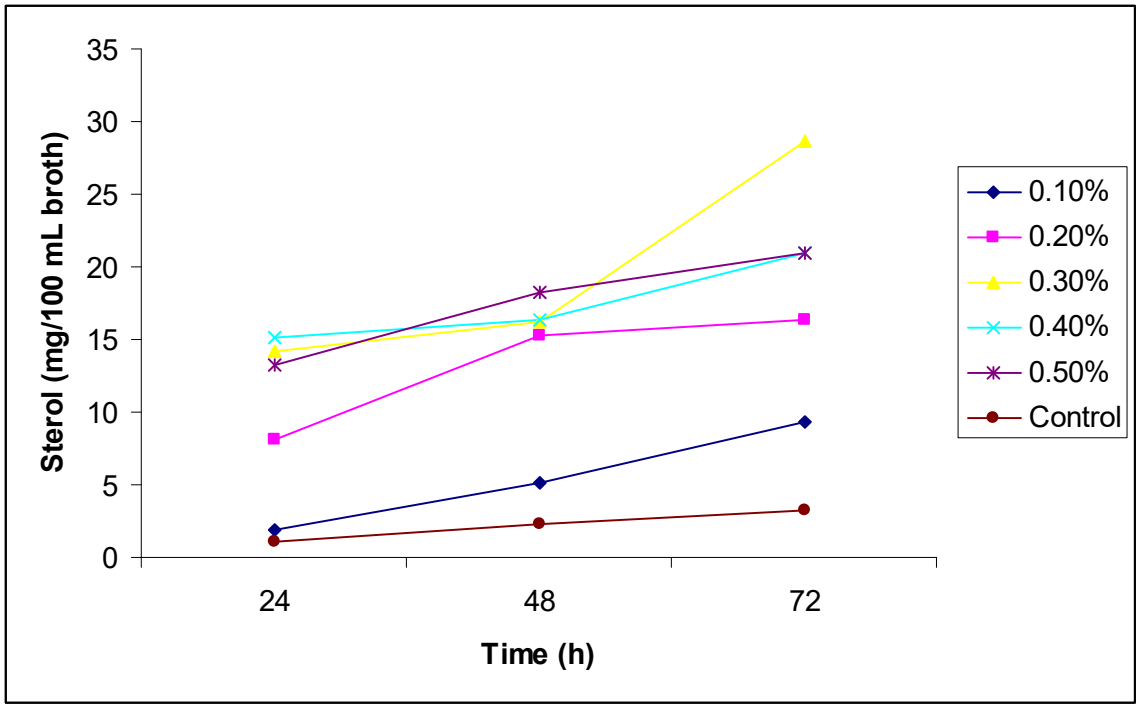

Fig. 2. Effects of sodium acetate supplementation on sterol production.