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Full Length Research Paper

Improvement and enhancement of clavulanic acid production in *Streptomyces clavuligerus* using vegetable oils

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Clavulanic acid (CA) is a potent inhibitor of β-lactamases. Oil can be used as a source of carbon and energy in CA production. To develop a policy for enhanced production of CA, the selection of a production medium and an optimum pH, different vegetable oils as carbon source have been used. The effects of different medium containing vegetable oil on cell growth and CA yield production during the fermentation of *Streptomyces clavuligerus* ATCC 27064 were demonstrated. In this study, three out of eight tested oils supported CA production. Medium containing olive oil showed two-fold higher CA yield than glycerol containing medium. The highest productivity was obtained at initial pH 7. We concluded that using olive oil as a sole source of carbon and energy for cultivation of *S. clavuligerus* is a promising strategy for CA production. It has several scientific advantages and economic benefits that lead to increased antibiotic titre and can be considered as a cheaper alternative compared to carbohydrates. The results of this study can be applied for the efficient production of β-lactamase inhibitory antibiotics.

Key words: Clavulanic acid, enzymes, β-lactamases, vegetable oils, *Streptomyces clavuligerus.*

INTRODUCTION

The most important mechanism for bacterial resistance to β-lactam antibiotics such as penicillins and cephalosporins is the production of β-lactamases (Bush, 1989; Bebrone et al., 2010). Varieties of pathogenic gram-positive and negative bacteria have the ability to produce β-lactamases which leads to inhibition of the antibiotic activity through the hydrolysis of the active β-lactam ring. Clavulanic acid (CA) acts as a potent inhibitor of the β-lactamases and has been used in combination with conventional β-lactam antibiotics (Butterworth, 1984; Hirakata. et al., 2009). The combination of CA with amoxicillin is the most common and efficient example with high levels of antibacterial activity used in the treatment of infectious diseases (Baggaley et al., 1997; Nagy, et al, 2010). CA can be produced industrially by the fermentation of *Streptomyces clavuligerus* ATCC 27064 strain, which requires a source of carbon, nitrogen and energy for the biosynthesis of cellular matter and products during normal cell operation, maintenance and production (Saudagar et al., 2008; Banos et al., 2009).

The most common carbon source that is generally used for the production of CA in *S. clavuligerus* fermentation is glycerol with arginine or soybean used as a nitrogen source (Chen et al., 2003; Teodoro et al., 2006; Ortiz et al., 2007; Banos et al., 2009). It has been reported that glycerol has been widely utilized as carbon source in the process with CA titres of up to 3.25 g/l (Chen et al., 2002;

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Abbreviations: CA, Clavulanic acid; **MOPS,** 3-(N-morpholino)-propanesulfonic; **TN,** total nitrogen; **HPLC,** high performance liquid chromatography.

Roubos et al., 2002). Although carbohydrates, including glycerol, are the uncomplicated energy sources for cell growth and secondary metabolite production, they lead to decrease in the rate of biosynthesis as a result of rapid catabolism (Saudagar et al., 2008; Perez-Redondo, et al., 2010). The best alternative to maintaining the level of glycerol for long period is substituting lipids for glycerol (Butterworth, 1984).

Utilization of glycerol by *S. clavuligerus* increases the possible use of carbon sources with low solubility such as oils to avoid carbon catabolite regulation. The addition of oil is preferred on an energy basis because typical oil contains about 2.4 times the energy of glycerol (Peacock et al., 2003). It can also act as antifoam and enhance secondary metabolism for the stimulation of bacterial growth and product synthesis (Large et al., 1998; Efthimiou et al., 2008). In addition, they are the cheapest available alternative carbon sources. According to Large et al. (1998), irrespective of the microorganism involved or the localization of the enzyme, the carbon source which is essential to lipase activity may act as stimulant or inhibitor to this activity. In the process of CA production by *S. clavuligerus* utilizing medium containing lipid and glycerol, the lipid consumption starts only after glycerol exhaustion, indicating that glycerol is a repressor of the lipase synthesis (Kim et al., 2007).

The addition of oil to growth medium has some disadvantages that occur due to the presence of higher residual oil level than that of carbohydrates, while oxygen requirement is higher for oil metabolism when compared to carbohydrates (Saudagar et al., 2008). Also, high residual oil levels may lead to increased medium viscosity and warrant additional downstream processing (Chen et al., 2002; Teodoro et al., 2006). Reduction in residual oil levels may further increase antibiotic titres and significantly reduce downstream processing costs. The high level of oil remaining may be due to physical limitation of oil mass transfer in the bioreactor or limitations due to the rate of lipid utilization (Large et al., 1999).

Since the initial pH influences the apparent decomposition of CA and pH changes also influence culture conditions for most fermentation processes (Parente et al., 1994), it is important to determine the optimal pH for cell growth and metabolite production (Peacock et al., 2003). A small number of studies have paid attention on the use of oil as a carbon and energy source for CA production by *S. clavuligerus*. Generally, these studies have used oil as substitute to other carbon sources, as well as using complex culture media containing other substrates such as flour, peptones and protein hydrolysates (Maranesi et al., 2005).

In the present work, the effect of different commercially available vegetable oils added as carbon source, on cell growth in *S. clavuligerus* cultures and supporting CA production were investigated. The results of this study should help in the development of novel strategies in improving the use of oil containing medium to enhance CA production.

MATERIALS AND METODS

Microorganism and cultivation conditions

Vegetative cells of *S. clavuligerus* ATCC 27064 (5.0 g/l dry weight) stored in cryotubes with 10% (v/v) glycerol at −70°C were used.

Culture media

In this work, reactivation medium for reactivation of the microorganism from the cryotubes and production medium for the preparation of inoculums and fermentation steps were used.

The seed medium used presented the following ingredients: Glycerol, 10 g/l; bacto peptone, 10 g/l; malt extract, 10 g/l; yeast extract, 1 g/l; K_2HPO_4, 2.5 g/l; $MgSO_4$ $7H_2O$, 0.75 g/l; $MnCl_2$ $4H_2O$, 0.001 g/l; $FeSO_4$ H_2O, 0.001 g/l; $ZnSO_4$ $7H_2O$, 0.001 g/l; MOPS buffer [3-(N-Morpholino)-propanesulfonic] acid (100 mM) 21 g/l. The medium was adjusted to pH 7 with 5 M NaOH solution prior to autoclaving at 121°C for 20 min. The medium used in the inoculum cultivation was equivalent in composition to the corresponding production culture medium as described later.

The production culture medium was based on that described by Maranesi et al. (2005) which present the following composition: 10 g/l of Starch; 20 g/l of soybean flour; 23 g/l of oil; and 1.2 g/l of phosphate in addition to 0.001 g/l $ZnSO_4.H_2O$, 0.001 g/l $FeSo_4$ $7H_2O$, 0.001 g/l of $MnCl_2$.4H2O of trace elements, and 1 ml/l of silicon antifoam. The medium was adjusted to pH 7 and sterilized at 121°C for 20 min. As the nitrogen present in the medium originates from the soybean flour, which contains around 50% of protein, the concentration of total nitrogen (TN) in the medium was 1.6 g/l. Glycerol was added to the medium before sterilization, while in the oil-containing experiments, oil was added to the autoclaved medium by sterile filtration through 0.2 µm hydrophobic Minisart SRP25 filters (Sartorius, Epsom, UK).

Cultivation conditions

A spore suspension (0.5 ml), which had been harvested from an agar plate, was added to a 100 ml baffled Erlenmeyer flask containing 20 ml of seed medium and incubated in a rotary shaker (New Brunswick Sci., model G-25, USA) at 28°C, 250 rpm, for 36 h.

For the experiments in a shaker, a quantity of 500 µl of this culture was added to 25 ml seed culture medium using two Erlenmeyer flasks of 250 ml and incubated in a rotary shaker at 28°C and 250 rpm for 24 h. This two-step preculture method ensured minimal carry over of rich nutrients in the production flasks. All cultures were submitted regularly to microscopical examination for disperse growth and contamination.

The culture obtained from the preculture mentioned earlier (50 ml) was inoculated into a 3 L flask containing 900 ml of production medium containing oil (olive, castor, coconut, cotton seed, corn, palm, sunflower or linseed oil). Then, 50 ml of this inoculated medium was transferred to each of the eight 500 ml Erlenmeyer flasks (each containing one of the oils). Cultivations were performed in incubating shaker at 28°C and 250 rpm for 140 h. Samples was drawn every 20 h and tested for the determination of cell growth and product concentrations after centrifugation at 3720 ×*g* and 5°C for 15 min. All cultures were performed in triplicate.

Analytical methods

Viscosity measurement

Cell growth was determined indirectly at different time intervals during the fermentation by measuring the apparent viscosity of the whole

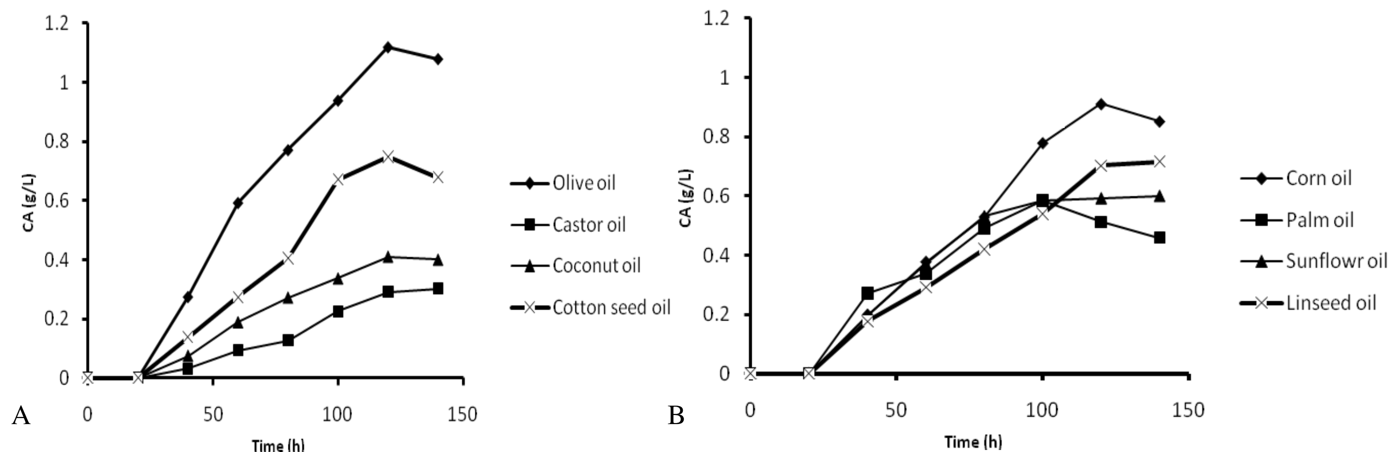

Figure 1. Effect of oils on clavulanic acid production in *S. clavuligerus.*

culture broth using Brookfield viscometer (Model DV-E, Middleboro, MA) at 20 rpm with spindle number 25 at 25°C.

CA content

CA produced during the fermentation was determined at pre-determined time intervals using high performance liquid chromatography (HPLC) as described by Foulstone and Reading (1982) which has been modified by Tabuck et al. (1985). CA in the form of lithium salt (SmithKline Beecham Pharmaceuticals) was used as standard.

Lipase assay

A sensitive method was used for the determination of lipase activity consuming tributyrin as substrate (Large et al., 1999) with a 842 titrando pH stat titrator and synthesis controller system (Metrohm AG, Herisau, Switzerland) at 30°C. Briefly, 10 ml of the broth was centrifuged at 5000 rpm for 25 min; the supernatant was washed with distilled water and resuspended in 10 ml distilled water. One milliliter of cell suspension was assayed with tributyrin as substrate. Automatic titration was carried out with 0.01 M NaOH.

RESULTS AND DISCUSSION

To enhance the production of CA, different oils containing media were used. The capability of *S. clavuligerus* ATCC 27064 strain to develop and produce CA in different media containing oil as carbon source was investigated in exploratory shake-flask cultures and compared with glycerol containing medium. The use of oil as a sole source of carbon in the production of CA as an alternative of glycerol has a positive effect and is proven to be a promising choice. The media containing oils were randomly classified into two groups A and B. Group A comprised of olive, castor, coconut and cotton seed oil, while group B comprised of corn, palm, sunflower and linseed oil.

CA production by *S. clavuligrus* and the cultivation media containing oils (groups A and B) are represented in Figure 1. In group A, cell growth and CA production started after 20 h of cultivation, still during the growth phase; however most of the production occurred later, after about 40 h. The production rate in most of the oils containing media decreased after 120 h. The same behavior was observed in group B cultivation media; however the results showed that the rate of CA production decreased after 100 h of cultivation in medium containing coconut oil in group A and palm oil containing medium in group B. CA formation accompanied by simultaneous decomposition has been reported. According to Chen et al. (2003), CA concentration dropped mainly due to the autolysis resulting from the rising pH, microbial decomposition and CA re-metabolization.

Due to the particulate nature of the culture medium and the hyphal growth of *S. clavuligerus*, methods of traditional biomass measurement, dry weight and optical density were not possible. Protein measurements were not used for biomass quatitation because of the presence of protein in the soyaflour added to the production medium. Cell growth rate was determined by measuring the viscosity of the culture medium (Figure 2). Viscosity data shows that maximum rates of cell growth were reached at 100 to 120 h.

Three out of eight tested oils supported good CA production. In group A, the highest CA concentration was observed in medium containing olive oil and cotton seed oil which yielded 1.12 and 0.749 g/l of CA, respectively. In group B, culture medium containing corn oil produced 0.911 g/l, whereas media containing castor and coconut oil in group A and sunflower and palm oils in group B did not support CA production. Although maximum cell growth was detected with other oils in comparison with corn and cotton seed oils, the rate of CA production was low. Product yield is considered as one of the most important parameters to assess the suitability of a culture medium used as a source of carbon because it depends on the metabolic pathways involved in the conversion of substrate into product (Westerhof and Kholodenko, 2004; Efthimiou et al., 2008). It was observed that olive oil

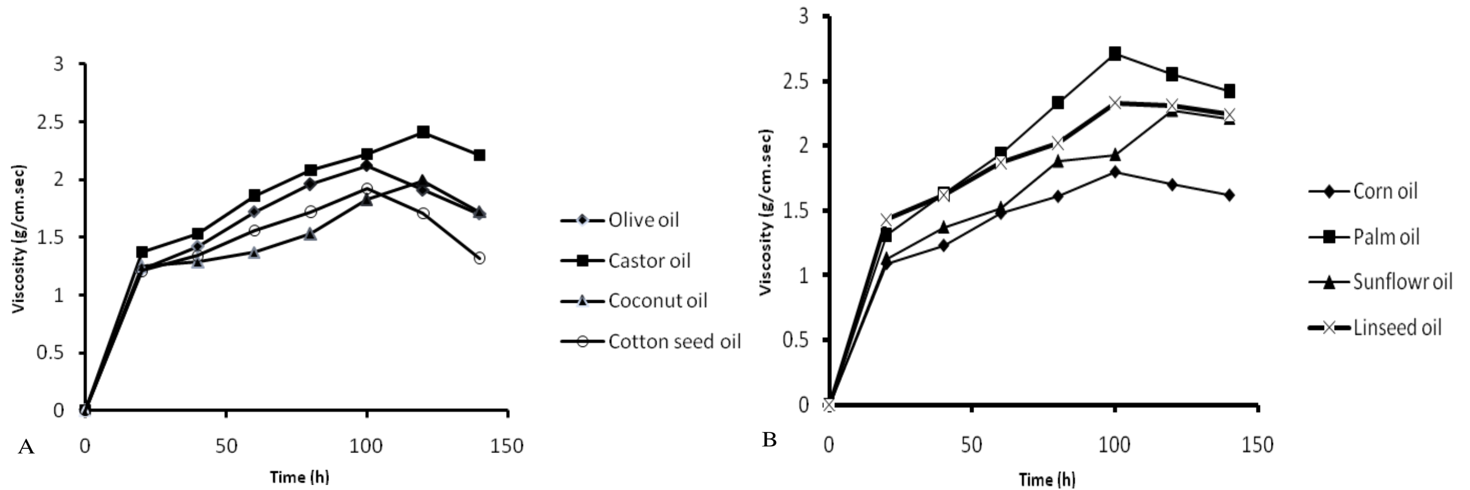

Figure 2. Viscosity of the culture media during clavulanic acid production.

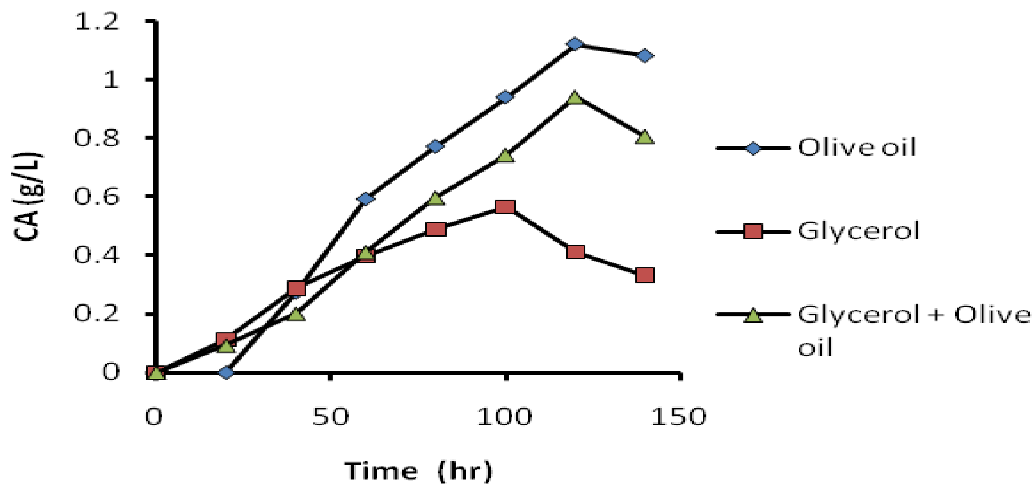

Figure 3. CA production in media containing olive oil, glycerol and combination of olive oil and glycerol (1:1).

containing medium produces higher CA than other oils. This consequence is in harmony with the results reported by Macris et al. (1996) and Baptista-Neto et al. (2000). These results show that CA production is not associated with microorganism growth. In our study, it was observed that the rate of CA production was proportional to the level of oleic acid content in the tested oils, so oils containing high percentage of oleic acid such as olive oil (78.1 %), corn oil (30.5 %) and linseed oil (18.5 %) gave the highest amount of CA concentration. Based on this result, oils containing higher percentage of oleic acid were very effective and efficient substrate to be used for increasing the production of CA. However this observation needs further investigation and more efforts to emphasize this fact.

Figure 3 represents comparison of CA production in media containing olive oil, glycerol and combination of glycerol with olive oil as carbon source. Production of CA in medium containing olive oil was delayed by more than 20 h in comparison with glycerol, but it gave about twofold higher CA production than glycerol medium (1.12 g/l). Glycerol uptake rate was faster than olive oil and was exhausted before 100 h, but in the case of olive oil culture, CA production continued up to 120 h. Moreover, CA production in glycerol medium began at 20 h and reached the maximum at 100 h, followed by a decrease in CA concentration. According to Efthimiou et al. (2008), the triglycerides in olive oil are hydrolysed by lipases produced by the bacteria to release glycerol and unesterified fatty acids into the culture medium that can then be taken up by the bacteria.

The medium containing combination of glycerol and olive oil (1:1) started in production, is faster than medium containing olive oil alone (20 h) and in the same time with

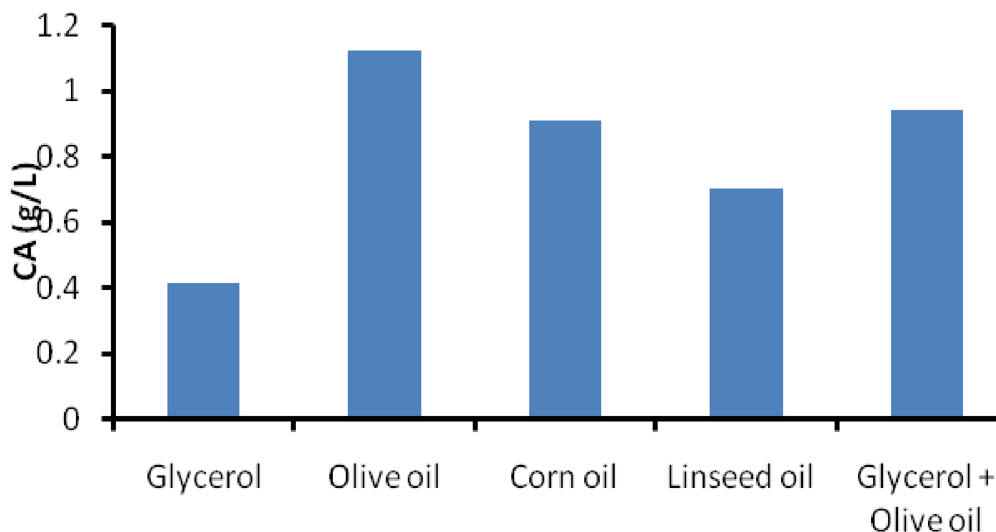

Figure 4. Different oils support for the CA production in *S. clavuligerus*.

medium containing glycerol. The overall CA production from the combination of glycerol and olive oil containing medium was higher than glycerol containing medium and lower than olive oil containing medium. As olive oil is hydrolyzed to glycerol and fatty acids by microbial lipase, it seems that the addition of more glycerol is not required and it reduces the CA production in accordance with those found in studies by Pitlik (1997). That could be due to the faster consumption of glycerol and the delay in the consumption of olive oil during fermentation. Large et al. (1999) reported that the reduction in residual oil levels increases the antibiotic titer and this result was established by Ortiz et al. (2007). It could be concluded that slow hydrolysis of olive oil was the limiting step of the process since all the glycerol generated by hydrolysis was completely consumed by the microorganism.

Figure 4 shows that olive oil containing medium gave the highest CA yield in comparison with different vegetable oils used in this study. Also, it gave slightly higher yield when compared with glycerol and olive oil containing medium. That could be due to the higher energy provided by olive oil compared to carbohydrates. This result indicated that olive oil is reliable with a medium and gave the highest production of CA in comparison with the study by Ortiz et al. (2007) and Efthimiou et al. (2008).

The effect of different initial pH (6, 7 and 8) on the production of CA using glycerol and olive oil containing media were investigated in this study. The study showed that at an initial pH 6 (Figure 5), a decrease in CA production occurred in olive oil containing medium, growth was markedly inhibited by acidity and the concentration of CA was obviously low (0.177 g/L) when compared with pH 7. The same behavior was observed in glycerol containing medium with over all CA production of 0.025 g/l. It was also clearly observed that pH 8 appeared to influence the production of CA from both olive oil and glycerol containing media which was slightly higher than that produced at pH 6. However, the optimum pH productivity of CA was obtained at an initial pH of 7 (1.12 and 0.564 g/) in both olive oil and glycerol containing media, respectively. This may be due to the slow hydrolysis of olive oil which was maintained during the cultivation. These results are comparable with the study of Large et al. (1999) as it was found that the smallest level of lipase activity in *S. clavuligerus* was observed in pH 7 and it is approximately three times lower than that in pH 7.4.

Enhanced CA production was achieved in the present work with medium containing 23 g/l of olive oil and 10 g/l soluble starch as mentioned before. Since the best production was attained with medium containing olive oil and starch, a new medium was prepared by replacing starch with more olive oil, so that the initial oil concentration was 28 g/l, to give the same amount of carbon in both media. Figure 6 shows the results obtained with the new medium. It was observed that the behavior was very similar to that found with starch containing medium. Maximum growth was achieved at 120 h and CA titre was around 0.997 g/l.

Conclusion

Using vegetable oils as a sole source of carbon can support bacterial growth and enhance the CA production, but a carefully choice of the oil is very essential other wise the CA yield will be significantly decreased.

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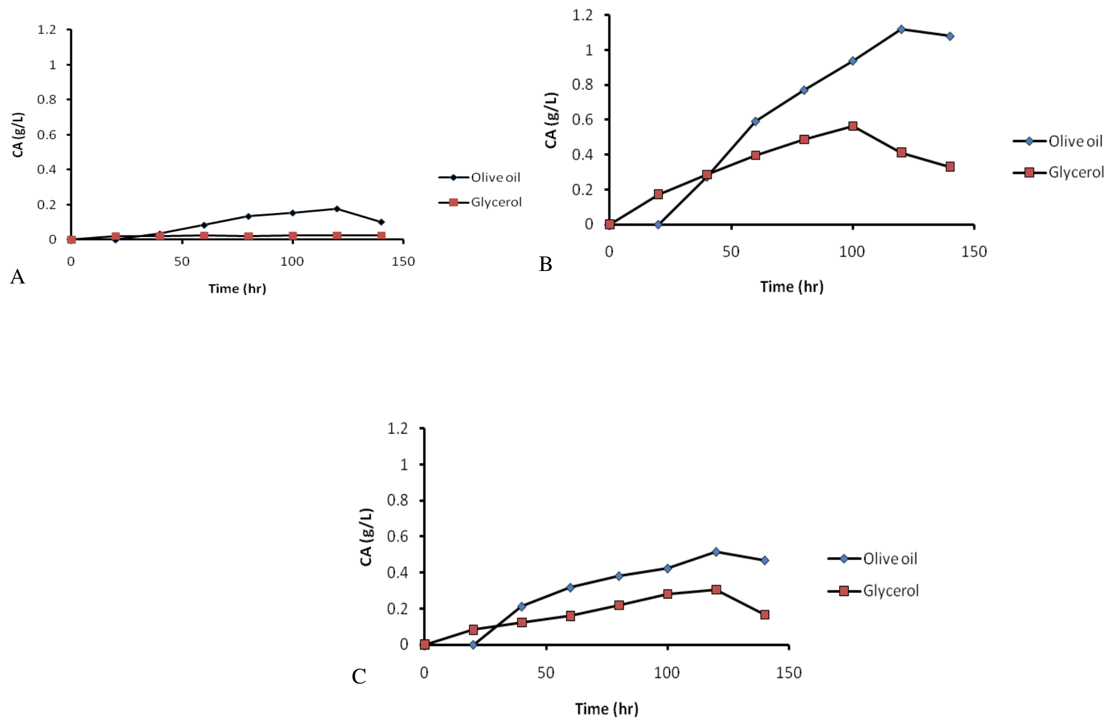

Figure 5. Effect of pH on CA production in *S. clavuligerus*. A, pH 6; B, pH 7; C, pH 8.

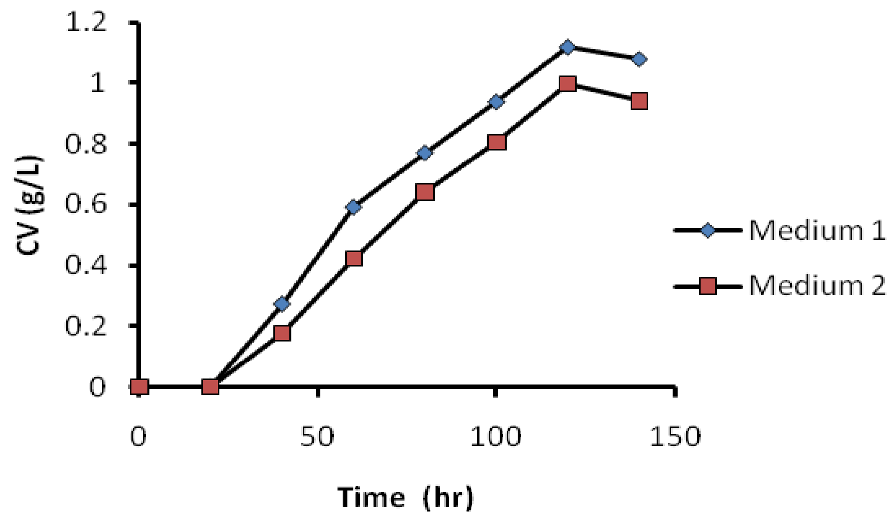

Figure 6. CA concentration from cultivation of *S. clavuligerus* in new medium.

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