ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (1): 138-143, 2009
ISSN 1682-296X

Bioprocessing and Scaling-up Cultivation of *Bacillus subtilis* as a Potential Antagonist to Certain Plant Pathogenic Fungi, III

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Abstract: The antagonistic and inhibitory activity of *Bacillus subtilis* isolate G-GANA7 (GenBank accession No. EF583053) obtained from Abo-Homos in Egypt, was tested against six fungal isolates belonging to four different genera, *Rhizoctonia solani*, *Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum*. The processing of *B. subtilis* isolate G-GANA7 was cultured in 3 L bench-top New Brunswick Scientific BioFlow III bioreactor for producing the maximum yield of biomass and antifungal compound. Fed-batch processes were automated through a computer aided data bioprocessing system AFS-BioCommand multi-process management program to regulate the cell growth rate by controlling interactively the nutrient feed rate, temperature, pH and agitation speed based on dissolved oxygen. In batch cultivation, the process suffered from low yield of cell mass (3.2 g L^{-1}) and antifungal activity because of high initial glucose concentration followed by acetate formation which the causal agent for inhibition of cell growth. Constant and exponential fed-batch strategies were adopted to circumvent this potential problem. Fed-batch cultivation of *B. subtilis* was conducted at the specific growth rate of 0.13 and 0.1 h^{-1} for constant and exponential strategies, respectively. High cell density of 12.8 and 14.6 g L^{-1} for both operations, with an overall biomass yield of 0.45 g g^{-1} was achieved. The inhibitory activity of antifungal in supernatant reached its maximum value of 2 and 2.2 cm for constant and exponential fed-batch cultivations.

Key words: *Bacillus subtilis*, biocontrol, batch cultivation, fed-batch cultivation and modeling

INTRODUCTION

The overuse of chemical pesticides has caused soil pollution and harmful effects on human beings. Accordingly, biological control of soil borne diseases has been attracting attention. Several strains related to *B. subtilis* produce insect toxins, peptide antibiotics and antifungal, some of which have been used in biocontrol and agricultural crop protection. Jiang *et al.* (2001) showed that *B. subtilis* was the most effective antagonist against *Peronophythora litchi*. Both the antagonist and its extract were effective in controlling artificially wound-inoculated fruit pathogens, but the use of the extract had better effect than that of the antagonist. Chitarra *et al.* (2003) found that the supernatant fluid of *B. subtilis* YM 10-20 inhibited germination of *Penicillium roqueforti* conidiospores. The germinating efficiency of *P. roqueforti* spores after 8 h of incubation in the absence of *B. subtilis* YM 10-20 supernatant fluid was 84%. In the presence of 10, 25 and 50% of supernatant fluid, the percentage of germination decreased to 7, 1 and 0%, respectively. The *B. subtilis* strain KS03 isolated from rice straw and identified as a biological control agent inhibited the anthracnose disease fungus *Gloeosporium gloeosporioides* (Cho *et al.*, 2003). The potential of *B. subtilis* GA1 to reduce post-harvest infection caused by *Botrytis cinerea* was tested on apples by treating artificially wounded fruits with endospore suspensions. Strain GA1 was very effective at reducing disease incidence during the first 5 days following pathogen inoculation and an 80% protection level was maintained over the next 10 days (Toure *et al.*, 2004). Fourteen *B. subtilis* isolates (B1 to B14) obtained from different Egyptian sites showed antagonistic and inhibitory effect

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against *Rhizoctonia solani*, *Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum* (Matar *et al*., 2008 (I)). In a fed-batch process, substrates and fresh media are fed into the reactor after depletion of glucose in the batch phase, but the harvesting is still done all at the end of the process. This type of process is used very often since it allows relatively high biomass or product concentrations to be achieved. These may not be achievable in batch because the total amount of needed substrate would be so much that the initial substrate concentration would be strongly inhibiting the process (Sonnleitner, 1998; Wirtz, 2002). Since both overfeeding and underfeeding of nutrients are harmful to cell growth and product formation, development of a suitable feeding strategy is important in fed-batch cultivation. Various feeding strategies have been developed to control the nutrient concentration within the optimal range and have been applied to high cell density culture of several microorganisms such as *Escherichia coli* (Lee, 1996). *B. subtilis* isolate G-GANA7 was selected from fourteen isolates for batch cultivation in bioreactor, the biomass achieved was 3.2 g L^{-1}. Inhibitory activity of supernatant was increased near the end of the stationary phase. The activity reached its highest value of 1.7 cm in death phase (Matar *et al*., 2008 (II)). The nutrients can be added at a constant feed rate. When the substrate is limited, the cells can grow linearly. The specific growth rate continuously decreases. When the substrate is in excess, the cells can grow exponentially. However, no quasi steady state is achieved (Yamane and Shimizu, 1984). Exponential fed-batch culture has been used widely in the high cell density culture of yeast and bacteria. Wilms *et al*. (2001) reported that 100 g L^{-1} cell dry weight was achieved by using exponential feeding strategy and 3.8 g L^{-1} recombinant L-N-carbamoylase in *E. coli* was produced using a positively regulated promoter.

The objective of this study was to isolate and identify bacterial isolates which have an antagonistic effect against certain plant pathogenic fungi, also for maximizing the production of biomass or the resultant inhibitory metabolic by-product using different fermentation strategies to use as a biocontrol agent.

MATERIALS AND METHODS

Bacterial and fungal isolates: *B. subtilis* isolate G-GANA7 (GenBank accession No. EF583053) was obtained from Abo-Homos in Egypt and identified according to the morphological, biochemical and physiological tests recommended by Sneath *et al*. (1986) and Collee *et al*. (1996), also according to 16S rRNA gene. Six fungal isolates belonging to four different genera were isolated in 2002-2003 from different hosts and area in Egypt, *R. solani*, *Helminthosporium* sp., *Alternaria* sp. and *Fusarium oxysporum*.

Antagonistic and inhibitory effect of *B. subtilis* isolate G-GANA7 (*in vitro*): Antagonistic effect of *B. subtilis* isolate G-GANA7 and inhibitory effect of supernatant fluid were performed against all fungal isolates according to Toure *et al*. (2004) and Chitarra *et al*. (2003). The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Fermentation experiments

Bioreactor: Fed-batch cultivations were performed in a 3 L bench-top bioreactor (Bioflow III, New Brunswick, N J, USA) equipped with two 6-bladed disc-turbine impeller and four baffles and connected to a digital control unit. The process was automated through a computer aided data bioprocessing system AFS-BioCommand multi-process management program. The set points for temperature and pH value were 30°C and 7, respectively and pH was controlled by automatic feeding of 2 N NaOH. Compressed air was supplied initially at 0.5-1.0 VVM (air volume per broth volume per min) through sterile filter. It can be manually controlled in parallel with agitation speed (150-900 rpm) to maintain the dissolved oxygen level above 20%. The dissolved oxygen level and pH values were controlled and measured on-line with METTLER TOLEDO electrodes. Antifoam A (Sigma) was used for eliminating foaming.

Fed-batch cultivation: Fed-batch process was started with batch phase with an initial volume of 1900 mL fresh Number 3 medium of which each 1 L contained 10 g peptone, 10 g glucose, 1 g KH_2PO_4 and 0.5 g $MgSO_4$. $7H_2O$ in distilled water and a pH of 6.8 (Asaka and Shoda, 1996) inoculated with 100 mL shake flask pre-cultured seeds. At the stationary phase and after depletion of glucose, feeding phase was started with addition of the feeding medium of which each 1 L contains 300 g glucose, 300 g peptone, 50 g KH_2PO_4 and 25 g $MgSO_4.7H_2O$. The strategy of feeding was studied using two different modes: constant feeding with a feeding rate of 0.27 mL min^{-1} and exponential predetermined feeding rate started from of 0.14 mL min^{-1} to final rate of 0.27 mL min^{-1} (Huang *et al*., 2004). During the time of cultivation, several samples of culture were taken and mass weight was determined by measuring the Optical Density (OD) at 550 nm. Glucose level was estimated by enzymatic (GOD-PAD) colorimetric kit (Diamond Diagnostic, Egypt) and the inhibitory activity assay of supernatant fluid was performed against *Alternaria* sp. according to Chitarra *et al*. (2003). Cell mass weights were determined from a calibration curve of

optical density at 550 nm (OD_{550}) of the fermentation broth versus cell dry weight. One unit of OD_{550} was found to be equivalent to 0.42 g L^{-1} dried cell weight.

RESULTS

B. subtilis isolate G-GANA7 (GenBank accession No. EF583053) had antagonistic effect against all tested fungi, but showed more antagonistic effect against *Alternaria* sp. (Fig. 1). Data was explained by Matar *et al.* (2008 (I)).

When fed-batch cultivation of *B. subtilis* isolate G-GANA7 (GenBank accession No. EF583053) was carried out in 3 L bench top bioreactor and biomass as assessed by optical density, concentration of consumed glucose and inhibitory activity of supernatant of isolate G-GANA7 were measured as a function of time, the results shown in Fig. 2 were obtained. In the batch phase, the culture grew after the lag phase, exponentially with a maximum specific growth rate of 0.3 h^{-1}. The culture was maintained at a higher value of dissolved oxygen which decreased gradually indicating cell growth and glucose consumption from the culture broth. The dissolved oxygen decreased due to the increasing in oxygen demand for growing the culture (Fig. 4). To guarantee a sufficient oxygen supply, oxygen was kept at above 20% by raising the agitation speed. The consumption of the total amount of glucose was used as an indication of ending the batch phase. After glucose depletion, the fed-batch phase started with addition of the feeding medium using constant feeding strategy with feed rate of 0.27 mL min^{-1}. The concentration of oxygen was limited to a value of 20% by means of agitation control. The cell was grown linearly after starting the feed stream with a specific growth rate of 0.13 h^{-1} (Fig. 3). The biomass achieved was 12.8 g L^{-1}. At the end of feeding phase, inhibitory activity of supernatant reached its maximum value of 2 cm (Fig. 2).

The results obtained from the application of the exponential feeding method are shown in Fig. 5 which shows biomass as expressed in optical density, the alteration in concentration of consumed glucose and inhibitory activity of supernatant of *B. subtilis* isolate G-GANA7 plotted as a function of time. In the batch phase, the culture grew with a maximum specific growth rate of 0.31 h^{-1}. Dissolved oxygen decreased gradually due to the increasing in oxygen demand for the growing culture. The oxygen was kept at above 20% (Fig. 7). After glucose depletion, fed-batch phase started with feeding of fresh medium using exponential feeding strategy of predetermined feed rate started from an initial rate of 0.14 mL min^{-1} to final rate of 0.27 mL min^{-1} (Fig. 7).

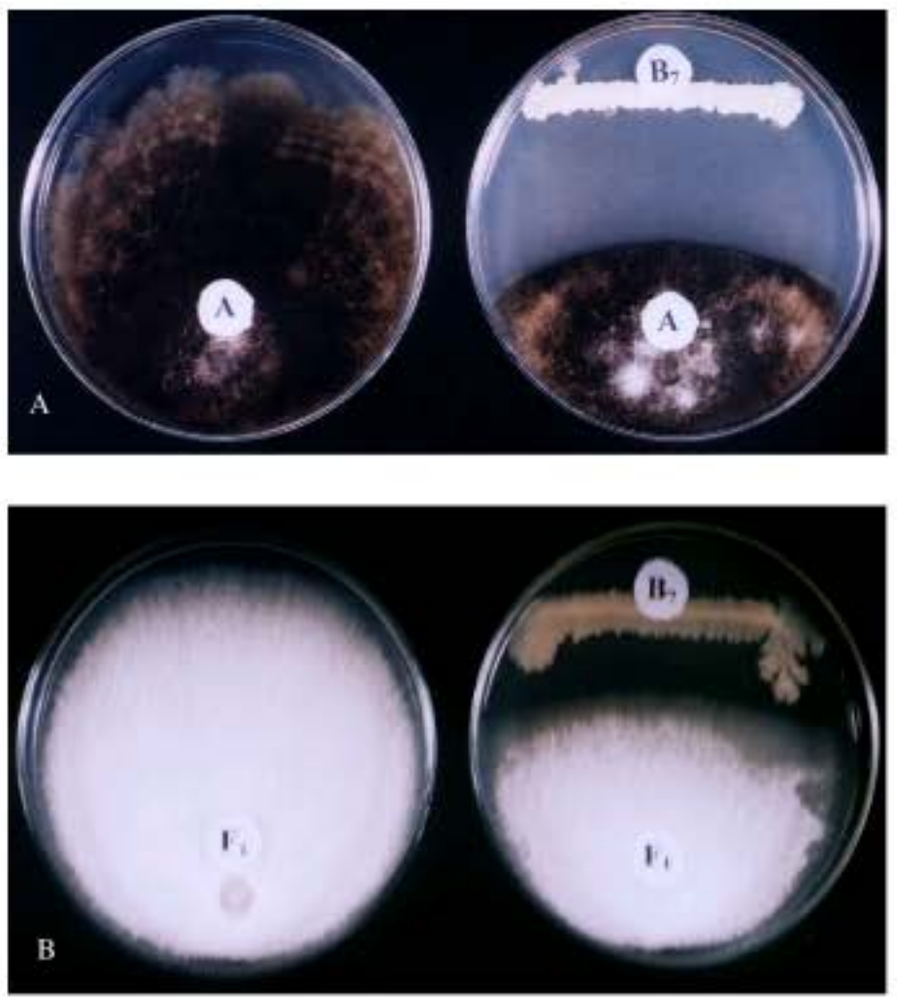

Fig. 1: Antagonistic effect of *Bacillus subtilis* isolate G-GANA7 against plant fungal pathogens: (A) *Alternaria* sp. and (B) *Fusarium oxysporum* isolated from tomato

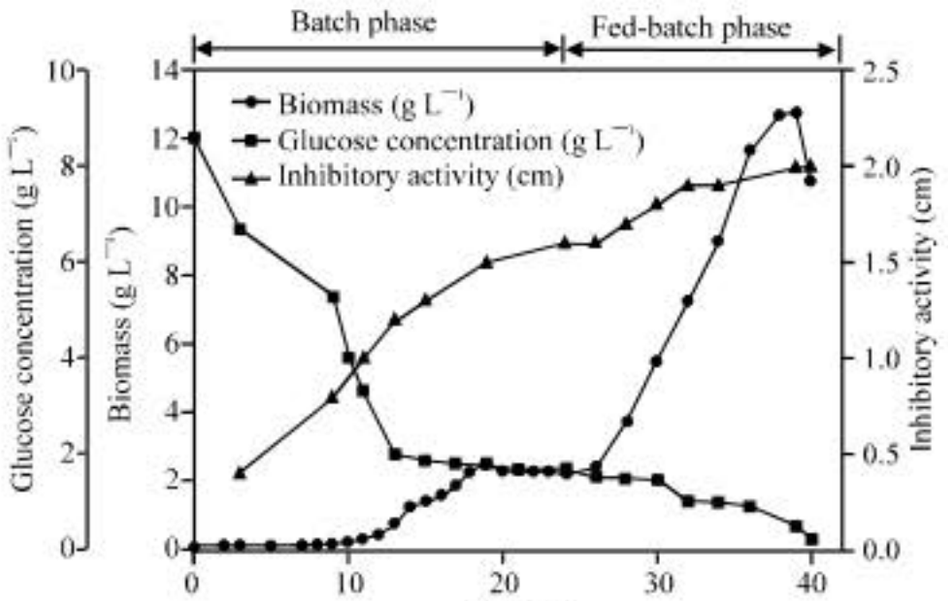

Fig. 2: Biomass, glucose concentration and inhibitory activity of supernatant as a function of time for high cell density constant fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

Compressed air was controlled manually in parallel with agitation speed (150-900 rpm) to maintain the dissolved oxygen concentration not below 20%. The cell was grown after starting the feed stream with a specific growth rate of 0.1 h^{-1} (Fig. 6). The biomass achieved was 14.6 g L^{-1}. At the end of feeding phase, inhibitory activity of supernatant reached 2.2 cm (Fig. 5).

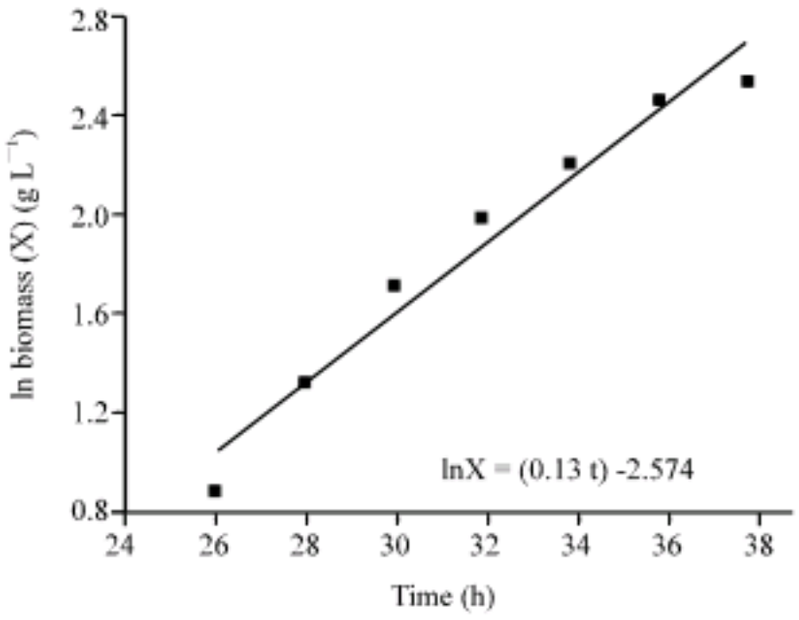

Fig. 3: The relationship between time (h) and ln biomass (X) in g L^{-1} in the feeding step for high cell density constant fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

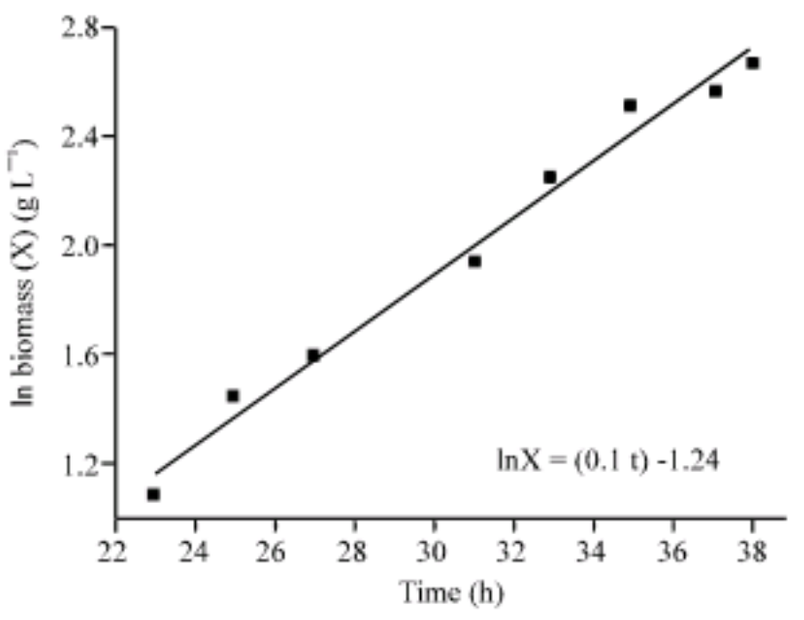

Fig. 6: The relationship between time (h) and ln biomass (X) in g L^{-1} in the feeding step for high cell density exponential fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

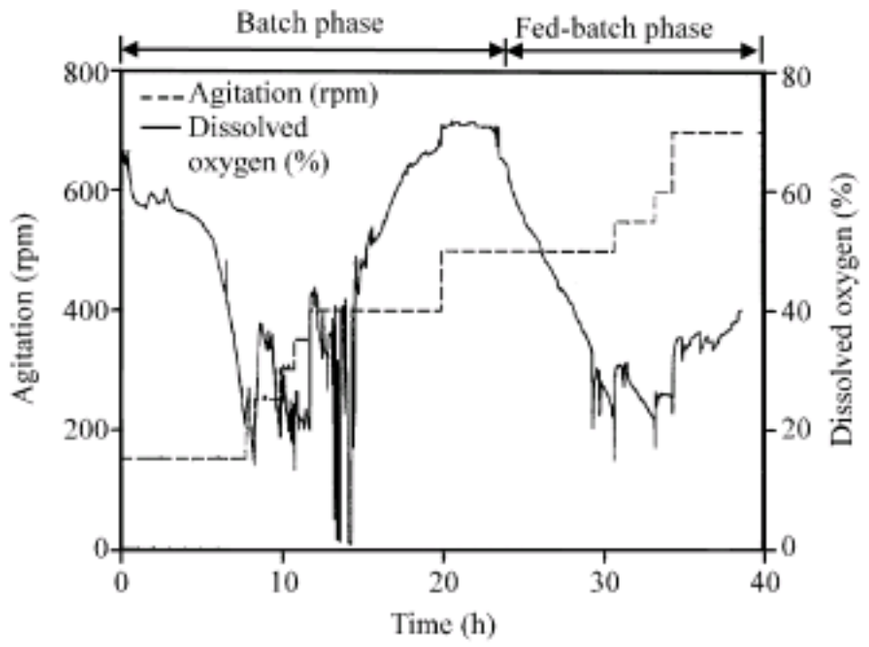

Fig. 4: Agitation and dissolved oxygen as a function of time during constant fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

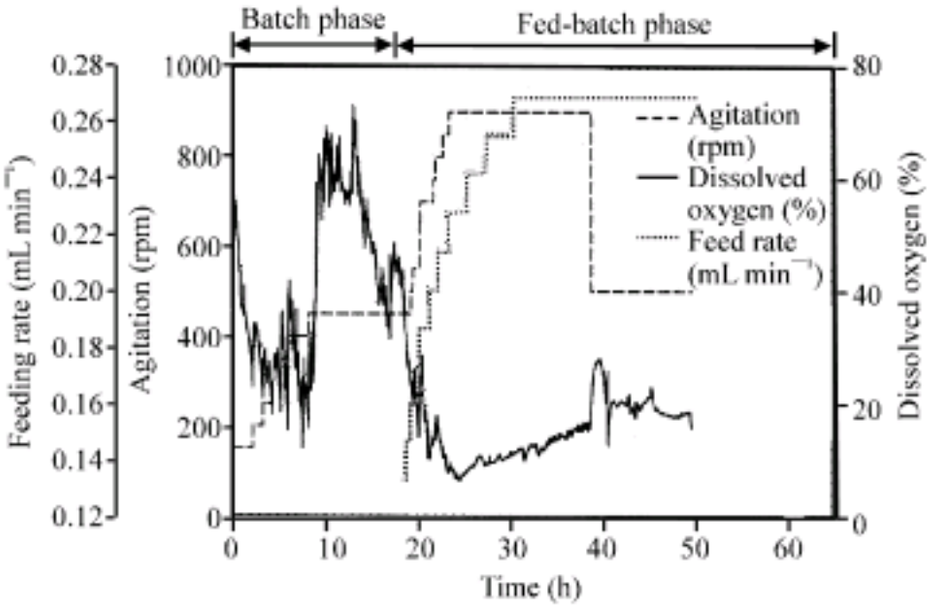

Fig. 7: Feeding rate (mL min^{-1}), agitation (rpm) and dissolved oxygen (%) as a function of time during exponential fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

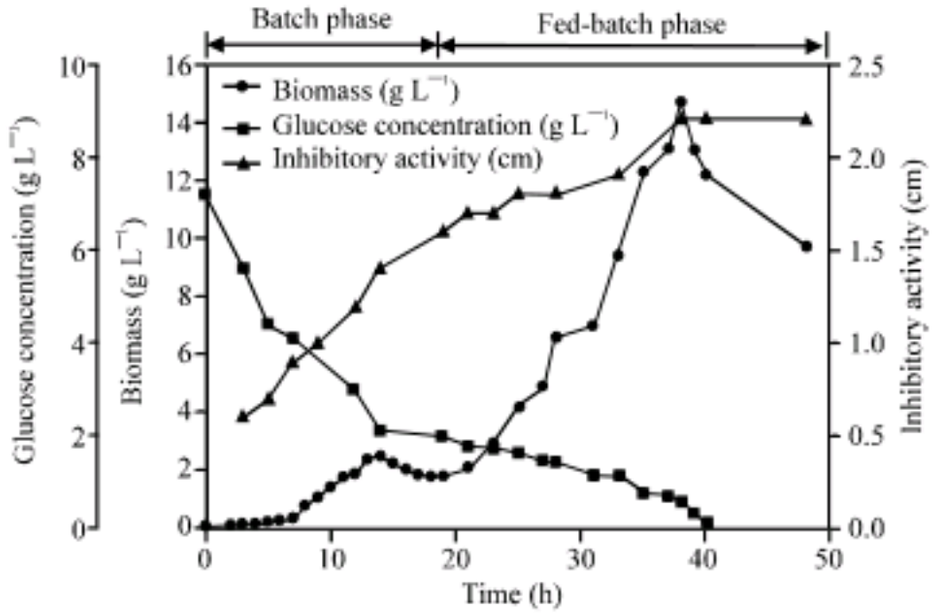

Fig. 5: Biomass, glucose concentration and inhibitory activity of supernatant as a function of time for high cell density exponential fed-batch cultivation of *Bacillus subtilis* isolate G-GANA7

Modeling of fed-batch fermentation: Fed-batch cultivation is described in basic biochemical engineering as the type of system where nutrient is added when its concentration falls below some set point. The nutrient is added in several doses, to ensure that there is not too much of the nutrient present in the bioreactor at any time. If too much of a nutrient is present, it may inhibit the growth of the cells. By adding the nutrient a little bit continuously, the reaction can proceed at a high rate of production without getting overloaded.

Feeding was carried out essentially as described by Huang *et al*. (2004).

If the specific growth rate μ is constant Eq. 1 is valid:

$$XV = X_0 V_0 e^{\mu t} \quad (1)$$

where, t is the time (h), V_0 is the broth volume when feed starts (L), V is the broth volume at time (L), X_0 is the cell mass concentration when feed starts (g L^{-1}) and X is the cell mass concentration in the broth at time (g L^{-1}).

Next, assume that the glucose amount in the broth is kept very low:

$$\frac{d(SV)}{dt} = 0 \quad (2)$$

where, S is glucose concentration in the broth (g L^{-1})
The mass balance equation for glucose is:

$$\frac{d(SV)}{dt} = FS_0 - \frac{\mu XV}{Y_{X/S}} \quad (3)$$

where, F is the feed rate (L/h), S_0 is glucose concentration in the feed (g L^{-1}) and $Y_{X/S}$ is the biomass yield on glucose (g/g).

Substituting Eq. 2 and 3 into Eq. 4 gives:

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{S_0 Y_{X/S}} \quad (4)$$

DISCUSSION

Antagonism is ubiquitous in nature among different species. For a long time, people have been interested in rationally making use of it in the areas of biological control of plant disease. Plant fungal diseases are difficult to control and can cause huge damage to economic crops. Environmental pollution, caused by abusing chemical biocides, is another serious problem. Using antibiotic producing bacteria to control plant fungal diseases is a popular topic and has extensively been studied (Raaijmakers *et al*., 2002). Compared with chemical biocides, many antibiotics produced by antagonistic strains have the advantage of being easily decomposed in nature, leaving no harmful residues behind. The results, obtained here, of *in vitro* sensitivity of phytopathogenic fungi to antagonistic bacteria revealed that *B. subtilis* isolate G-GANA7 was suppressive to the tested isolates of phytopathogenic fungi, are consistent with those obtained by others (Wagih *et al*., 1989; Gong *et al*., 2006).

Two fed-batch fermentation strategies were performed to increase the overall biomass and to obtain high concentrations of products compared to batch fermentation by minimizing substrate inhibition and accumulation of inhibitory by-products such as acetic acid and propionic acid which is the most harmful by-product for the *B. subtilis* (Park *et al*., 1992), or to avoid oxygen insufficiency. Accumulation of acetic acid is known to be a function of many factors including anaerobic condition, high partial pressure of carbon dioxide, high glucose concentration or high specific growth rate (Lee, 1996).

High cell density culture is an attractive means of achieving high concentration of final product. However, researchers cannot obtain high cell density (greater than 10 g L^{-1}) with batch culture alone, because cells suffer from substrate inhibition and by-products inhibition. Yee and Blanch (1993) documented that catabolic acid by-products would accumulate in the fermentation broth during batch culturing causing inhibition of cell growth and production of products. In order to attain high cell densities by the use of glucose as carbon source, fed-batch culture is preferred to minimize acetate production and to eliminate substrate inhibition.

Exponential fed-batch cultivation gave higher cell mass and inhibitory activity of supernatant than constant fed-batch cultivation. Cell mass and inhibitory activity of supernatant in exponential fed-batch cultivation were 14.6 g L^{-1} and 2.2 cm, respectively but the corresponding figures for constant fed-batch cultivation were 12.8 g L^{-1} and 2 cm. This may be referred to substrate inhibition and accumulation of acetate in exponential fed-batch cultivation were low compared to constant fed-batch cultivation, hence the cells were grown with a low specific growth rate of 0.1 h^{-1} in exponential fed-batch cultivation and the consequent reduction of the glucose concentration while the specific growth rate in constant fed-batch cultivation was 0.13 h^{-1}. Akesson *et al*. (2001) minimized acetate formation in *E. coli* cultures using controlling of the feeding rate of glucose. In the early stage of cultivation, the cell density is rather low, so the glucose feed rate is low due to the low glucose consumption rate. In this period, no acetate is formed. As cells grow, the feeding rate and oxygen consumption can be increased and finally the oxygen consumption exceeds the maximum oxygen transfer capacity even when the stirrer speed is at its maximum and an anaerobic condition will occur, so the feed rate should be reduced.

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