Production of Gibberellic Acid by Multiple Fed-batch Cultivation of *Gibberella fujikuroi*

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The growth of *Gibberella fujikuroi* and kinetics of gibberellic acid production was studied in a multiple fed-batch cultivation process at pH 5.0 and 30 °C. Application of four nutrient addition cycles of fed-batch mode of operation was used to extend the batch fermentation and increase the final mass concentration of biomass and gibberellic acid. The lag period was totally eliminated in the subsequent feeding of 25 % nutrient feeding and simultaneous withdrawal of the fermentation broth. At the end of the fourth cycle, a biomass concentration of $\gamma = 12.2$ kg m^{-3} and gibberellic acid mass concentration of $\gamma = 1.31$ kg m^{-3} were obtained. This cultivation methodology enhanced significantly the growth and production phase of the culture under non-limiting and non-inhibitory conditions, and may be performed on a continuous basis until the batch is contaminated.

Key words:
Multiple fed-batch fermentation, Gibberellic acid, *Gibberella fujikuroi*

Introduction

Gibberellic acid (GA_3) is a well-known growth promoting plant hormone with applications in agriculture, horticulture and the brewing industry. Chemically, GA_3 is a tetracyclic dihydroxy γ-lactonic acid having an empirical formula of $C_{19}H_{22}O_6$. Industrially it is produced by submerged fermentation using Ascomycetous fungus *Gibberella fujikuroi*[1,2] mainly due to its high fermentation rates. Although solid-state and immobilized fermentation techniques have also been applied to improve the product formation and productivity, several difficulties have been encountered such as scale up of the production to an industrial level, control of process quantities (pH, temperature and moisture), and the problem of maintaining sterility.[3,4] The use of immobilized cell encounters severe oxygen limitations and risk of strain mutation during continuous operations.[5] These techniques therefore could not be practiced on a large scale and for a longer period. The net result is that to date, the cost of GA_3 has been high enough to preclude its extensive use for plant growth promotion, except for certain high value plants. Reduction in its production cost via alternative bioprocessing strategies can lead to wider applications to a variety of crops.[6] The production of GA_3 in the batch submerged fermentation process features catabolic repression and substrate inhibition.[7,8] Application of the fed-batch culture technique particularly to substrate inhibited cultivation leads to significant process improvements. Fed-batch fermentations have been attempted in the past by using different arbitrary (trial & error) nutrient feeding strategies to improve GA_3 productivity.[9,10] Model based nutrient feeding strategies for improvement of GA_3 productivity have also shown improved product formation and productivity.[11] The objective of this investigation was to study the growth of *Gibberella fujikuroi* and gibberellic acid production under multiple fed-batch mode of cultivation, and demonstrate the semi-continuous mode of operation, which features no lag phase and enhances product formation phase under non-limiting, non-inhibitory cultivation conditions.

Materials and methods

Microorganism

Gibberella fujikuroi NRRL2284, procured from Northern Regional Research Laboratory, Peoria USA, was used in this study. The culture was maintained on potato dextrose agar slants at 4 °C and subcultured every month.

Inoculum preparation and culture medium

The inoculum was grown at 30 °C for 30 h on a rotary shaker at $n = 200$ min^{-1} in $V = 0.25$ dm^3 Erlenmeyer flask, containing 0.05 dm^3 media of

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following composition (in kg m^{-3}): $\gamma_{Glucose} = 30.0$, $\gamma_{NH_4NO_3}$=1.65, $\gamma_{MgSO_4 \cdot 7H_2O}$=5.0, $V_{corn\ steep\ liquor}$= 1.5 cm^3.[11] The pH of the medium was adjusted to 5.0 by NaOH (80 kg m^{-3}). The above cultivation conditions featured exponential growth of the inoculum. $\varphi = 10$ % inoculum was used for fermenter study. In the fermenter, the following medium composition[13] was used (in kg m^{-3}): $\gamma_{Glucose} = 80.0$, $\gamma_{NH_4NO_3} = 0.75$, $\gamma_{MgSO_4 \cdot 7H_2O} = 1.5$, $\gamma_{KH_2PO_4} = 3.0$, and $\gamma_{rice\ flour} = 2.0$. The initial pH of the medium was adjusted to 5.0 by NaOH (80 kg m^{-3}).

Batch cultivation

A $V = 3$ dm^3 laboratory fermenter (with ADI 1010 Biocontroller, Applikon Dependable Instruments, The Netherlands) with a working volume $V_w = 1.8$ dm^3 was used. The culture broth was agitated at $n = 700$ min^{-1} by two 6-flat blade turbine impellers. The temperature was controlled at 30 $\pm$ 1.0 °C. One dm^3 dm^{-3} min^{-1} oxygen supply was maintained through the ring sparger. The pH was maintained at 5.0 $\pm$ 0.25 by adding NaOH (80 kg m^{-3}) / HCl (73 kg m^{-3}). The samples were withdrawn at regular time intervals and analyzed for biomass, residual total nitrogen, glucose, lipid, extracellular polysaccharide, and gibberellic acid mass concentrations.

Multiple fed-batch cultivation

Multiple fed-batch cultivation was conducted in a 3-dm^3 fermenter with working volume of $V_w = 1.8$ dm^3. The operating conditions were similar to batch cultivation (as described in section 2.3). One-fourth volume (25 % of the working volume) of the culture was withdrawn from the reactor at different time intervals when the glucose mass concentration was significantly reduced and dissolved oxygen concentration indicated abrupt increase. The same (25 % of working volume) volume of the fresh medium (having optimum C/N ratio of 120) was quickly fed to the bioreactor. Four cycles of culture withdrawal and nutrient addition were successfully implemented.

Analytical procedures

For cell dry mass estimation, 0.005 dm^3 sample was centrifuged at $n = 5000$ min^{-1} for 15 min. The pellet was washed twice with distilled water and transferred to preweighed aluminium cups. The cells were dried to a constant mass at 80 °C in an oven. Glucose was estimated by the dinitro salicylic acid (DNS) method.[14] The total nitrogen concentration in the medium was determined by the Kjeldahl method.[15]

Estimation of gibberellic acid

The gibberellic acid was estimated by HPLC using Nova Pack C-18 column at 45 °C using a UV detector at wavelength (λ) of 254 nm. The flow rate of the mobile phase was $Q = 0.0005$ dm^3 min^{-1}. The mobile phase consisted of 30 % methanol containing $c = 0.01$ mol dm^{-3} phosphoric acid (pH 3.0 adjusted by KOH). The samples were initially filtered through 0.45 μm Millipore filters and adjusted to pH 2.5 with $\varphi = 10$ % HCl.[16]

Estimation of lipid

The cell free broth was extracted three times with hexane and the extracted samples kept in an oven at 60 °C for the evaporation of solvent. After drying, the lipid fraction was quantified by dry mass determination.[17]

Estimation of extracellular polysaccharide

The cell free broth was precipitated with ethanol [Ψ = 2:1] in the presence of 1 % KCl and the samples were incubated at 4 °C for 24 h. After incubation, the precipitated polymer was separated by centrifugation and quantified by dry mass determination.[18]

Result and discussion

Batch cultivation

The batch kinetic profiles of biomass, pH, residual substrates and products are shown in Fig. 1.

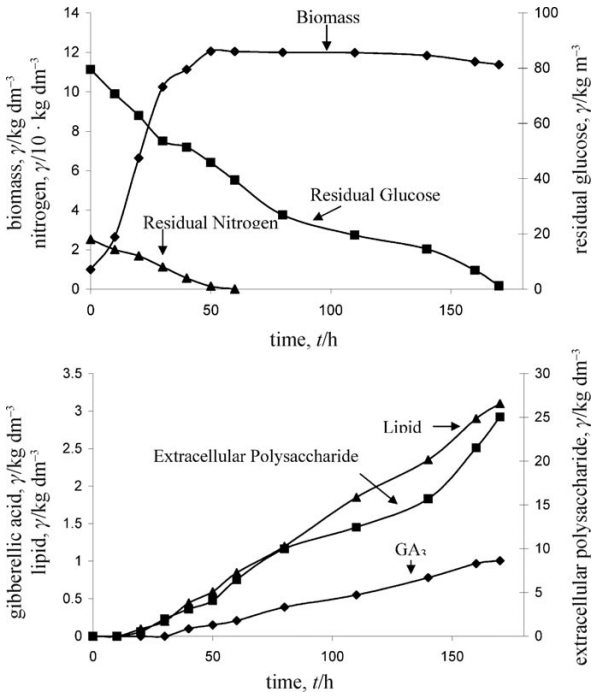
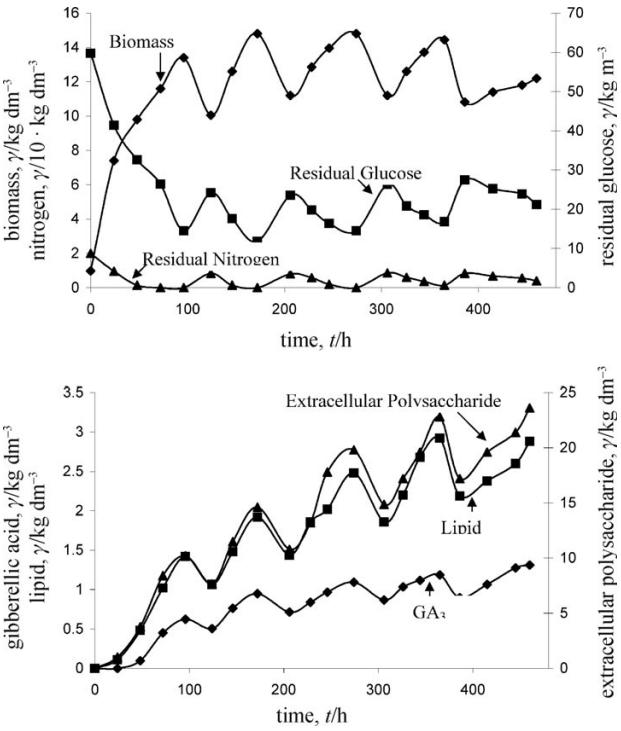


Fig. 1 – *Batch gibberellic acid cultivation by* G. fujikuroi *in a 3-dm^3 fermenter*

The growth and GA_3 production were further increased when *G. fujikuroi* was cultivated in a 3-dm^3 fermenter under controlled pH (5.0 ± 0.25) conditions. The culture started growing almost instantaneously after inoculation without a lag phase. The maximum biomass concentration of 12 kg m^{-3} was obtained in 50 h. During this period, about 80 % total nitrogen was consumed, whereas about 30 kg m^{-3} glucose was metabolized (out of initial concentration of 80 kg m^{-3}), however, no Gibberellic acid was formed. Gibberellic acid production started only after 30 h, before the exhaustion of the nitrogen source and reached a final mass concentration of 1 kg m^{-3} during 40 to 170 h. This was significantly higher than the GA_3 concentration (0.18 – 0.67 kg m^{-3}) reported in the literature for submerged cultivation.[19,20,21] It has been reported in the literature that, during the production of GA_3 by *G. fujikuroi,* some other side products such as lipids and polysaccharides have also been produced.[17] The lipid and extracellular polysaccharide production started after the lag phase of the culture and continued till the end of fermentation (170 h) and their maximum mass concentrations were 3.1 kg m^{-3} and 25.05 kg m^{-3} respectively.



F i g . 2 – *Multiple fed-batch cultivation by* G. fujikuroi *in a 3-dm^3 fermenter. Feedings 1 to 4: Removal of fermentation broth and addition of fresh medium (25 % of the working volume)*

Multiple fed-batch cultivation

The product formation phase of batch fermentation could be extended by converting it into multiple fed-batch fermentation by feeding fresh substrate as and when required (very nicely indicated by abrupt dissolved oxygen increase). The multiple fed-batch kinetic profiles of biomass, residual substrates and products are shown in Fig. 2. The cultivation was continued under batch mode for 96 h after which the first feeding started when the glucose concentration was significantly reduced to 14.5 kg m^{-3}. A portion of the culture (25 %) was withdrawn and supplemented by fresh medium in the bioreactor. This ensured the benefit of high inoculum ratio (75 %) at the time of additional fresh feed and immediate continual production of Gibberellic acid (which otherwise was not possible in batch). Four cycles of culture withdrawal and addition of fresh medium were carried out. At the end of the fourth cycle a biomass concentration of 12.2 kg m^{-3} and GA_3 mass concentration of 1.312 kg m^{-3} were obtained (as opposed to 1 kg m^{-3} in batch). Feeding of fresh medium after withdrawal of the culture broth decreased the inhibitory effect of accumulated toxic products and the product itself. This might have resulted in the enhancement of enzyme activity involved in the GA_3 pathway. The maximum mass concentration of lipid and extracellular polysaccharide obtained was 2.88 kg m^{-3} and 23.6 kg m^{-3} respectively at the end of the fourth cycle, which is lower than the values obtained in batch fermentation. The mass concentration of GA_3 was maximal in the fourth feed cycle indicating that, the more the number of nutrient feed cycles, the better the final mass concentration of GA_3 in the broth. The mass of total GA_3 produced in multiple fed-batch was found to be 3.24 g as opposed to 1.8 g GA_3 in batch fermentation. This indicates that multiple fed-batch fermentation is a better option than batch fermentation as it reduces the non-productive downtime of the reactor for cleaning, refilling, sterilization, etc.

Conclusion

This study shows an appreciable increase in GA_3 mass concentration by four cycles of feeding fresh medium (25 % of working volume) and simultaneous withdrawal. It was possible to eliminate the lag period for GA_3 product formation. Increasing the feeding cycle (4th cycle) resulted in enhancement of gibberellic acid concentration. The production phase continued from 50–450 h of cultivation. It may therefore be possible to further enhance the concentration by increasing the cycle of nutrient feeding in such multiple feed fed-batch cultivation.

List of symbols

c – concentration, mol dm^{-3}
n – stirring speed, min^{-1}
Q – volume flow rate, dm^3 min^{-1}
T – temperature, °C
t – time, min, h
V – volume, dm^3
V_f – volume of fermenter, dm^3
V_w – working volume, dm^3
γ – mass concentration, kg m^{-3}
φ – volume fraction, %
Ψ – volume ratio

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