Bioresource Technology 100 (2009) 374–379

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant *Fusarium moniliforme* cells immobilized on loofa sponge

Samir A. Meleigy, Mahmoud A. Khalaf *

Radiation Microbiology Department, National Center for Radiation Research and Technology (NCRRT), P.O. Box 29, Nasr City, Cairo, Egypt

ARTICLE INFO

Article history:
Received 9 April 2008
Received in revised form 11 June 2008
Accepted 12 June 2008
Available online 5 August 2008

Keywords:
Gibberellic acid
Fusarium moniliforme
Milk permeat
Immobilization
Loofa sponge

ABSTRACT

Gibberellic acid (GA) production from milk permeate was studied by 28 mutants of *Fusarium moniliforme*, among which mutant γ-14 was selected as the best producer.

Experiments were carried out in shaker flasks and fermentative process was analyzed with free and immobilized cells. Immobilization of mutant γ-14 cells onto loofa sponge discs was studied with respect to the optimization of the incubation temperature, initial pH, inoculum size (number of discs) and its reusability for GA production. Best yield of GA (2.40 gl^{-1}) was recorded by immobilized cells under optimized cultural conditions (4 immobilized discs, 30 °C and pH 5).

Data obtained during four reusable cycles showed high stability of GA production and reduction in the initiation time of acid production, resulting in higher levels of GA in shorter time duration. Immobilization of mutant γ-14 cells onto loofa sponge discs, permitted repeated reuse under the specified fermentation conditions for GA production from milk permeate.

1. Introduction

Gibberellins are a large family of structurally related diterpenoid acids that occur in green plants and some microorganisms. Gibberellic acid (GA) is an important member of gibberellins family and acts as a natural plant growth hormone, controlling many developmental processes such as the induction of hydrolytic enzyme activity during seed germination, stem elongation, induction of flowering, improvement of crop yield, overcoming dwarfism, etc (Tudzynski, 1991). Due to these properties GA has a wide application in agriculture, nurseries, tissue culture, tea garden, etc. Industrially GA is produced throughout the world by submerged fermentation (SmF) technique using the ascomycetous fungus *Gibberella fujikuroi* (recently named *Fusarium fujikuroi*) the perfect stage of *Fusarium moniliforme* (Bruckner and Blechschmidt, 1991). The cost of GA has restricted its use to preclude application for plant growth promotion, except for certain high value plants. Reduction in its production costs could lead to wider application fields (Shukla et al., 2005). The important factor in obtaining high yields of GA is the carbon source employed. Also, GA production, like that of several other secondary metabolites is initiated by the limitation (depletion) of nitrogen source from the medium (Shukla et al., 2003).

In Egypt, milk permeate is a cheap carbon source available in plenty as a dairy by-product, which has not been put to any economical use and it is discharged into dairy effluents. Inspection of a typical composition of milk permeate reveals that it is reasonably high in lactose and contains minerals necessary for microbial growth, but it is low in nitrogen (Yellore and Desai, 1998). Also, improvement of the microbial producer strain (through subjecting the genetic material to physical and chemical mutagenic agents) offers the greatest opportunity for cost reduction without significant capital outlay (Stanbury et al., 1995; Lotfy et al., 2007). This is achieved when a selected strain can synthesize a higher proportion of the product using the same amount of raw materials.

Immobilized biocatalysts such as enzymes, microorganisms, organelles, and plant and animal cells are being explored in the search for yield increase in the biotechnology processes. Recently, the immobilization of whole growing cells has been studied, mainly because of its economical potential. Advantages of an immobilized system based on living cells include their active metabolic ability to synthesize various useful and complicated bioproducts using the multi-enzyme steps, and the regeneration capability to prolong their catalytic life (Vignoli et al., 2006). Immobilization of microorganisms with a number of techniques including encapsulation, entrapment in polymer gels, and adhesion onto the surface of carriers has been reported (Aykut et al., 1988; Mozes and Rouxhet, 1984). Of these various methods adhesion to carriers has the advantage of simple preparation involving low cost procedures and the preservation of viability and activity. When compared with encapsulation or entrapment in polymer gel matrices, the immobilization by cell adhesion also has advantages in a lower diffusion limitation in the transport of

* Corresponding author. Tel.: +202 7571621; fax: +202 2749298.
E-mail addresses: samirelmeligy@hotmail.com (S.A. Meleigy), dr01khalaf@hotmail.com (M.A. Khalaf).

0960-8524/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2008.06.024

substrates, products, and oxygen (Saudagar et al., 2008). The major disadvantages of the encapsulation and entrapment techniques are release of the cells due to weak binding to carriers and are expensive (Hermesse et al., 1987).

Numerous matrices constructed from synthetic polymers or biological materials have been used for the immobilization of biocatalysts (Furusaki and Seki, 1992). Some of the synthetic matrices may be toxic (Macaskie et al., 1987). Biogels on the other hand, lack open spaces to accommodate new cell growth which then result in their rupture and the release of cells into the medium, also their efficiency is limited by diffusion restrictions, and decreased enzyme activity beside it is expensive (Iqbal et al., 1993).

A matrix for immobilization should ideally be strong, resistant to operating conditions, and preferably have an open structure as well as inexpensive. The plant-derived loofa sponge (*Luffa cylindrica*) is an inexpensive and easily available biological, and therefore, renewable, matrix produced in most of the tropical and subtropical countries. Merits of the loofa sponge as biomatrix include freedom from materials that might be toxic to microbial cells, simple application and operation technique, and a high stability during long-term repeated use (Iqbal et al., 1993; Ogbonna et al., 1997). The high void volume, permeability, and low cost of fibrous matrices make it particularly attractive. The simplicity of the immobilization technique, the strong binding and the low cost of the loofa sponge can help to find future applications for whole cells immobilization for various applications. Recently, the viability of using loofa sponge as a carrier for microbial cells was studied successfully (Vignoli et al., 2006; Saudagar et al., 2008).

To the best of our knowledge there are no reports on immobilization of *F. moniliforme* on loofa sponge for GA production. The objective of the present study was to produce fungal biomass immobilized loofa sponge discs to ascertain semi-continuous method for production of GA by a selected mutant of *F. moniliforme* from milk permeate.

2. Methods

2.1. Microorganism

F. moniliforme (local isolate) conserved in potato dextrose agar (PDA) slants at 4 °C and subcultured every two months was used in this study.

2.2. Preparation of spore suspensions

Spore suspension of fungal isolates were prepared by washing 10-days old cultures slants with sterile saline solution (0.9% NaCl) and shaking vigorously for 1 min. Spores were counted by plate count method and the count was adjusted to about 10^7 spores ml^{-1}.

2.3. Mutation and selection

Spore suspension (3 ml in sterile test tube) was exposured to sublethal dose of gamma rays (6.5 kGy, sufficient dose for 99.999% death of survival spores) and mutant colonies were selected based on variation in colony morphology (diameter, shape, color and pigmentation) and/or sporulation (heavy or weak) on PDA plates. Irradiation was carried out at the National Center for Radiation Research and Technology (NCRRT), using ^{60}Co gamma irradiation source of gamma chamber (4000A) with a dose rate 0.1 kGy min^{-1} at the time of the experiment. Purified mutated colonies were screened for GA production in milk permeate medium.

2.4. Preparation of milk permeate medium

Milk permeate was obtained from Agriculture Research Center (Giza, Egypt). The pH of the milk permeate was adjusted to 4.5 by adding 1 N HCl to remove the excessive proteins in it (Yellore and Desai, 1998). After filtration, the solution was boiled and cooled and excess protein was removed. The clear solution formed (which containing total sugar 65 gl^{-1} and nitrogen content 4.1 gl^{-1}) was adjusted to pH 6.0 with 1 N NaOH and divided into 50 ml in 250 ml Erlenmeyer flasks. The whey in flasks was autoclaved at 121 °C for 10 min, and it was used as a complete production medium of GA.

2.5. Inoculum preparation and batch cultures

One ml of inoculum about 10^7 spores ml^{-1}, was grown at 30 °C for 48 h on shaker incubator at 100 rpm in 250 ml Erlenmeyer flask, containing 50 ml seed medium of the following composition (in gl^{-1}): glucose, 30.0; NH_4NO_3, 1.65, $MgSO_4 \cdot 7H_2O$, 5.0; corn steep liquor, 1.5 ml. The pH of the medium was adjusted to 5.0 by 1 N NaOH. The above cultivation conditions featured exponentially growing cell population and a high concentration in inoculum. The sterile milk permeate medium (GA production medium) was inoculated with 2% (v/v) of inoculum in shake flasks. Cultures were then incubated at 30 °C and shaked at 150 rpm for the requisite time. Biomass was isolated by filtration, and supernatants were separated for necessary analyses.

2.6. Immobilization of F. moniliforme on loofa sponge discs

Method developed by Saudagar et al. (2008) for production of clavulanic acid by immobilized *Streptomyces clavuligerus* cells on loofa sponge, was modified and used in this study. Loofa sponge was used as an immobilization matrix and obtained from the ripped dried fruit of *Luffa aegyptica*. The loofa, free from seeds, was cut into discs of approximately 18–20 mm diameter and 2–4 mm thick, soaked in boiling water for 30 min, washed thoroughly with tap water, and left for 24 h in distilled water. The discs were then oven dried at 70 °C and sterilized by autoclaving at 120 °C for 20 min.

The spore suspension (10^7 spores ml^{-1}) of *F. moniliforme* was inoculated into four different 250 ml Erlenmeyer flasks each containing 50 ml of autoclaved seed culture medium with 2, 4, 6 and 8 pre-weighed loofa sponge discs, respectively. Flasks with no loofa sponge disc in the medium, were inoculated with 1 ml of spore suspension (10^7 spores ml^{-1}) to provide free biomass controls. The inoculated flasks were shaken at 100 rpm at 30 °C. After 48 h, both free and loofa immobilized biomass of *F. moniliforme* were harvested from the medium washed with sterile distilled water, and transferred to the GA production medium.

2.7. Effect of culture conditions

The shake flask experiments were primarily conducted to select the optima culture conditions that supported the maximum GA production. The effect of inoculum size, temperature (20–40 °C), initial pH (3–8) and reuse inoculum on GA production by the selected mutant immobilized on loofa sponge, were studied.

2.8. Analysis

The dry weight of the biomass was determined by weighting oven dried (70 °C overnight) separated free mycelia or immobilized sponge discs.

The gibberellic acid was extracted and estimated by the method of Kahlon and Malhotra (1986). Filtered fermented production medium (10 ml) was transferred to a centrifuge tube and 0.5 ml

zinc acetate (1 M) solution was added and shaken for 3 min, followed by addition of 0.5 ml potassium ferrocyanide solution (1 M) and the mixture was centrifuged for 15 min. Following centrifugation, 2.5 ml supernatant was transferred to a 250 ml flask containing 8 ml absolute alcohol and 90 ml HCl (30%). For control, 35 ml HCl solution (5%) was taken in a 250 ml flask and the volume made to 100 ml with distilled water. The flasks were incubated at 20 °C in a water bath for 75 min and the absorbance was read at 254 nm in a UV/Visible spectrophotometer (type: Helios Gamma). The GA concentration was obtained from a standard GA curve. Residual sugar in filtrate was measured by the phenol sulphuric acid method (Southgate, 1976) using glucose as the standard.

2.9. Statistical analysis

The means of three replicates and standard error (SE) were calculated for all the results of the GA obtained, and the data were subjected to analysis of variance (Spatz, 1993).

3. Results and discussion

3.1. Mutagenesis and screening for GA hyperproduction

In a screening experiment, the parental culture of *F. moniliforme* and its γ-irradiated colonies were grown on the basal production medium (milk permeate) for 6 days and compared with respect to GA and biomass production. As shown in Table 1, most of the mutagenized colonies, were improved with respect to GA production. The isolates γ-2, γ-11, γ-14, γ-19 and γ-28 showed marked GA overproduction records. Among those, maximum GA production was achieved by the mutagenized isolate γ-14 (0.86 gl^{-1}) with approximately 2.1 fold increase when compared to the wild type. Six successive cultures of this mutant, γ-14 isolate, on seed culture plates were further examined under the same fermentation conditions. All cultures showed approximately the same GA concentration as well as biomass production records indicating the stability of the characters of this isolate. Several previous studies recorded that the low doses of gamma radiation may stimulate the microbial metabolic activities (El-Batal and Khalaf, 2003; Khalaf and Khalaf, 2005).

3.2. Production by immobilized cells

Fed batch cultivation for production of GA by immobilized *F. moniliforme* cultures have been reported (Kahlon and Malhotra, 1986; Lu et al., 1995). In this study, in order to obtain high productivity of GA, a newly introduced carrier, loofa sponge was used for immobilization process

In the present study a less expensive and environmental friendly support material (loofa sponge) was utilized for immobilization of *F. moniliforme* γ-14 cells for GA production. The comparison of free and immobilized *F. moniliforme* γ-14 cells for GA production is presented in Table 2. Best yield of GA (1.2 gl^{-1}) with conversion rate 2.54% and productivity rate 8.33 $mgl^{-1}\,h^{-1}$ were obtained by immobilized cells, respectively, after 6 days. The yield of GA production by immobilized *F. moniliforme* cells in sodium alginate gel was more than that produced by free cells (Kahlon and Malhotra, 1986). The higher yield with an immobilized system depends upon the nature of material matrix, which affects the permeability of the cell towards high penetration of the substrate, as well as faster removal of the end products from the fermentation sites, due to protection of the mycelium from microenvironmental changes by the material matrix (Kahlon and Malhotra, 1986; Lu et al., 1995). GA productivity by *G. fujikuroi* cells immobilized with the carrier-covered copolymer of hydrophilic hydroxyethyl acrylate and hydrophobic trimethylpropane triacrylate (HEA-A-TMPT) was higher than that in the free cells (Lu et al., 1995; Saudagar et al., 2008). The results observed also explain that in contrast to the sodium alginate immobilization which requires more sophisticated equipments involving high cost, the more robust immobilized loofa sponge systems can be made simply by adding spore suspension to the medium containing the inexpensive sponge disc without any prior chemical treatment.

3.3. Effect of immobilized inoculum size

It was observed that, there was no increase in the immobilized loofa sponge disc weight in seed culture medium after 48 h (data not shown). This may be indication of the saturation of space for further immobilization on the disc. While the immobilized cells are packed tightly within the sponge, there remain large number of micro channels for free movement of the solute during the

Table 1
GA production from milk permeate by *F. moniliforme* parental isolate, and its mutant isolates

F. moniliforme isolates	Biomass (gl^{-1})	Consumed sugar (gl^{-1})	GA (gl^{-1})[a]	GA conversion (%)	GA productivity ($mgl^{-1}\,h^{-1}$)
Parental isolate × mutant isolates	6.72	36.5	0.41 ± 0.001	1.12	2.84
γ-2	6.42	40.2	0.52 ± 0.012	1.29	3.61
γ-5	5.65	38.6	0.45 ± 0.001	1.16	3.13
γ-8	6.14	40.1	0.49 ± 0.002	1.22	3.40
γ-11	6.70	44.2	0.77 ± 0.002	1.74	5.34
γ-14	7.32	46.4	0.86 ± 0.003*	1.85	5.97
γ-19	6.48	42.5	0.72 ± 0.003	1.69	5.00
γ-21	5.43	38.3	0.43 ± 0.001	1.12	2.98
γ-24	5.81	39.2	0.46 ± 0.001	1.17	3.19
γ-28	6.60	42.7	0.75 ± 0.004	1.75	5.20

*Significant from all GA values ($P < 0.05$).
[a] Values are means of 3 replicates ± SE.

Table 2
GA production from milk permeate by loofa sponge immobilized cells of *F. moniliforme* γ-14 (48 h age)

State of inoculum	Biomass (gl^{-1})	Consumed sugar (gl^{-1})	GA (gl^{-1})[a]	GA conversion (%)	GA productivity ($mgl^{-1}\,h^{-1}$)
Free cells (2%,v/v)	7.26	45.8	0.85 ± 0.012	1.85	5.90
Immobilized cells (2 discs/flask)	8.35	47.2	1.20 ± 0.018*	2.54	8.33

*Significant from GA value of free cells ($P < 0.05$).
[a] Values are means of 3 replicates ± SE.

fermentation process (Iqbal et al., 2005), thus making the conditions favorable for diffusion of media nutrients to the immobilized cells. Hence, 48 h old *F. moniliforme* γ-14 immobilized loofa sponge discs were used for the production of GA.

Immobilized inoculum (48 h old) in varying concentration (2–8 discs) was tested to find the optimum immobilized fungal mycelium for GA production. The best yield of GA (1.90 gl^{-1}) with conversion level 3.38% and productivity rate 13.20 $mgl^{-1}\,h^{-1}$ were obtained with 4 immobilized discs (Fig. 1). At immobilized inoculum greater than 4 discs (6–8) the production of GA was inferior. This may be due to resistance of the substrate to diffuse into the immobilized discs and product out of the discs. With 6 and 8 discs of inoculum size, utilization of sugars was maximum (61.4 and 63.70 gl^{-1}, respectively) but the yield of GA was low (0.88 and 0.47 gl^{-1}, respectively), this means that the microorganism utilized the sugars for vegetative growth. Kahlon and Malhotra (1986) entrapped *F. moniliforme* in various alginate gel concentrations and obtained low yields of GA with increase in gel concentrations due to limited transportation of oxygen to the inside and biocatalyst, thus lowering the efficiency of biocatalyst. Similar results were observed for sorbitol and clavulanic acid production by microbial immobilized cells on loofa sponge (Vignoli et al., 2006; Saudagar et al., 2008)

3.4. Effect of temperature

The effect of different temperature levels (20–40 °C) on the ability of *F. moniliforme* γ-14 isolate to transform milk permeate sugars

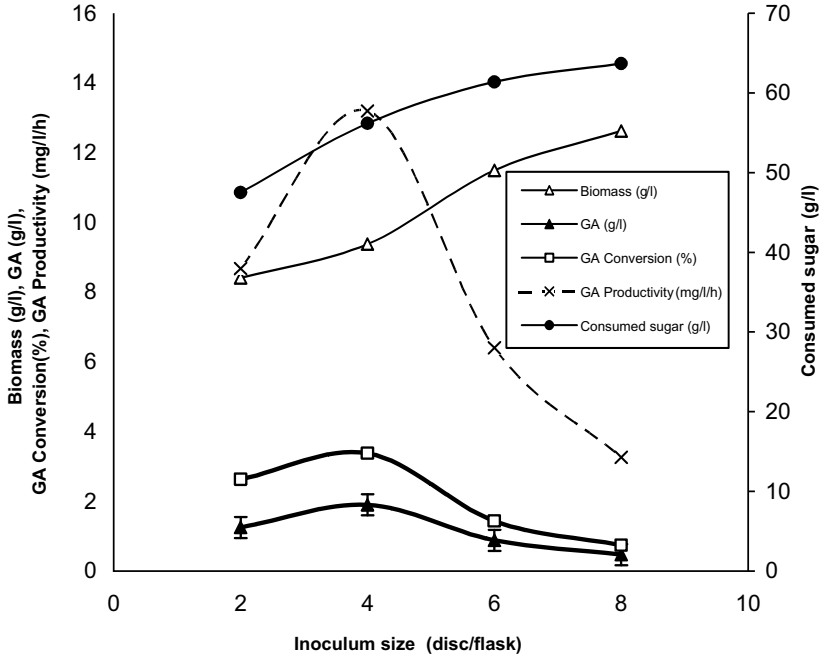

Fig. 1. Effect of immobilized inoculum size on GA production from milk permeate by loofa sponge immobilized cells of *F. moniliforme* γ-14.

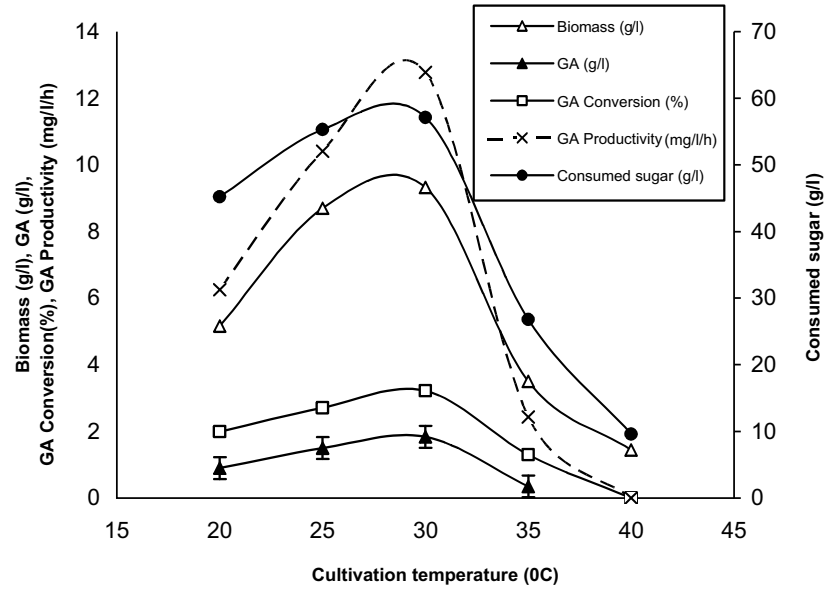

Fig. 2. Effect of culture temperature on GA production from milk permeate by loofa sponge immobilized cells of *F. moniliforme* γ-14.

into GA is presented in Fig. 2. Maximum GA (1.84 gl^{-1}) was produced at 30 °C by utilizing 57.1 gl^{-1} sugars. This value of GA was significant ($p < 0.05$) from the yield of GA produced at other temperature degrees. The yield of GA production decreased at 20, 25 and 35 °C to 0.9, 1.5 and 0.35 gl^{-1}, respectively. This may be due to change in enzyme activity at different temperatures or denaturation of the enzyme at high temperatures. Similarly, Kahlon and Malhotra (1986) found that the maximum yield of GA production by *F. moniliforme*, entrapped in alginate gel, was recorded at 25 ± 1 °C. According to the same author the proteins fold tightly at lower temperatures and thus decrease the catalytic activity. Also, Escamilla et al. (2000) recorded that the greatest production of GA in fluidized bioreactors by immobilized *G. fujikuroi* mycelium in Ca-polygalacturonate was at 30 °C. Complete inhibition of GA synthesis in our study was recorded at 40 °C (Fig. 2).

3.5. Effect of initial pH

The effect of initial milk permeate pH on GA production (Fig. 3) indicates that significant increase of GA production (2.25 gl^{-1}, $p < 0.05$) with conversion 3.64% and productivity rate 15.63 $mgl^{-1}\,h^{-1}$ were obtained at pH 5 after 6 days of incubation. Lower yields of GA production were obtained at pH 3.0 (0.36 gl^{-1}) and 8 (0.21 gl^{-1}). Also, initial pH 5.0 was recorded as more suitable pH range for GA production by immobilized *G. fujikuroi* mycelium (Escamilla et al., 2000). Meanwhile, Kahlon and Malhotra (1986) observed the high yield of GA production by immobilized *F. moniliforme* at initial pH 5.5. This lower yields of GA production at extreme pH values (pH 3 and 8, in this study) may be due to conformational changes and biocatalyst denaturation.

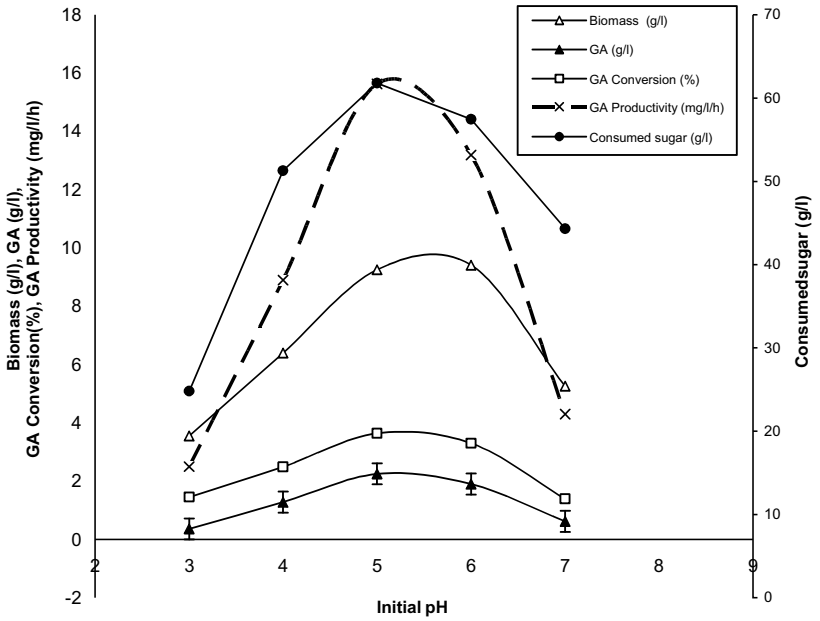

Fig. 3. Effect of initial whey pH on GA production from milk permeate by loofa sponge immobilized cells of *F. moniliforme* γ-14.

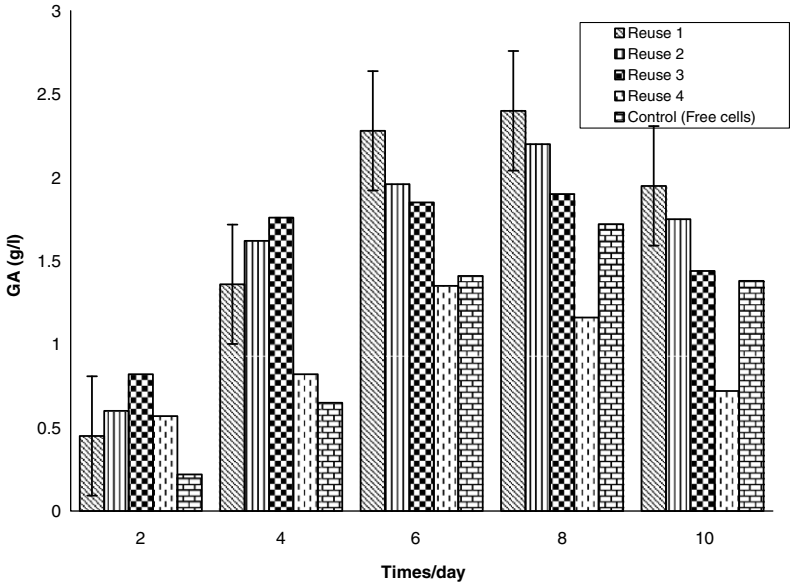

Fig. 4. Reusability of *F. moniliforme* γ-14 cells immobilized on loofa sponge disc for GA production from milk permeate.

3.6. Semi-continuous production of GA

Fig. 4 illustrates the reusability of *F. moniliforme* γ-14 isolate cells immobilized on loofa sponge discs for GA production from milk permeate. GA production in this experiment was estimated at five different time intervals viz., 2 d, 4 d, 6 d, 8 d and 10 d. Each replacement of production medium was designated as a reuse, and hence a semi-continuous mode of operation. GA production was studied up to four reuse cycles. During the first reuse the GA production increased gradually with the increase in time of fermentation and reached a maximum of 2.40 gl^{-1} at the end of 8 d. For the second reuse, the cells were already in the late exponential phase and hence a faster initiation of the GA production was observed. Levels of GA produced in second ruse were 0.60 and 1.62 gl^{-1} at the end of 2 and 4 d, respectively, as compared to 0.45 and 1.36 gl^{-1} for similar time intervals in the first reuse. These results illustrated that the mutant γ-14 isolate immobilized on loofa sponge discs can be repeatedly reused under the fermentation conditions. Although, a decrease in the final production level of GA was observed with every reuse, the time for initiation of GA production was decreased as the cells are already in the production stage after the first cycle. The decrease in the final production level may be as a result of increase in the number of immobilized dead cells with reuse. On the other hand, Lu et al. (1995) found that during 12 batch fermentation cycles over 84 days, GA productivity by immobilized *G. fujikuroi* cells with copolymer HEA-A-TMPT, was maintained at a constant value. This suggested that the immobilized cells produced a stable system for GA production.

4. Conclusion

From the previous results, it could be concluded that the mutagenized *F. moniliforme* γ-14 isolate could be effectively used for the production of GA from milk permeate. It was found that GA production from milk permeate with immobilized mycelia of mutant γ-14 on loofa sponge could reach 2.4 gl^{-1}, more than 2.8-times greater than previously reported by free cells with possibility to expand the production to four cycles. However, it is necessary to further optimize the fermentation conditions to achieve the demands of large scale GA production from this γ-irradiated potent isolate.

References

Aykut, G.G., Hasirci, V.N., Alaeddinoglu, G., 1988. Immobilization of yeast cells in acrylamide gel matrix. Biomaterials 9, 168–172.

Bruckner, B., Blechschmidt, D., 1991. The gibberellin fermentation. Crit. Rev. Biotechnol. 11, 1163–1192.

El-Batal, A., Khalaf, M., 2003. Wheat bran as a substrate for enhanced thermostable alpha-amylase production by gamma irradiated *Bacillus megaterium* in solid state fermentation. Egypt. J. Rad. Sci. Appl. 16, 443–464.

Escamilla, E.M., Dendooven, L., Magana, I.P., Parra, R.S., De la Torre, M., 2000. Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. J. Biotechnol. 76, 147–155.

Furusaki, S., Seki, M., 1992. Use and engineering aspects of immobilized cells in biotechnology. Adv. Biochem. Eng. Biotech. 46, 161–185.

Hermesse, M.P., Dereppe, C., Bartholome, Y., Rouxhet, P.G., 1987. Immobilization of *Acetobacter aceti* by adhesion. Can. J. Microbiol. 34, 638–644.

Iqbal, M., Saeed, A., Edyvean, R.G.J., Sullivan, B.O., Styring, P., 2005. Production of fungal biomass immobilized loofa sponge (FBILS)-discs for the removal of heavy metal ions and chlorinated compounds from aqueous solution. Biotechnol. Lett. 27, 1319–1323.

Iqbal, M., Shah W.A., Zafar, S.I., 1993. Biostructural materials: Novel supports for cell immobilization. In: Proceedings of International Symposium on Biotechnology for Sustainable Development. December 15–20, pp. 393–400.

Kahlon, S.S., Malhotra, S., 1986. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate. Enzyme Microb. Technol. 8, 613–616.

Khalaf, M., Khalaf, S., 2005. Pomegranate peels: A novel agro-residue for tannase production by gamma irradiated *Aspergillus versicolor* under solid state fermentation. Isotope Rad. Res. 37, 1005–1022.

Lotfy, W.I., Ghanem, K.M., El-Helow, E.R., 2007. Citric acid production by a novel *Aspergillus niger* isolate: I. Mutagenesis and cost reduction studies. Bioresource Technol. 98, 3464–3469.

Lu, Z.X., Xie, Z.C., Kumakura, M., 1995. Production of gibberellic acid in *Gibberella fujikuroi* adhered onto polymeric fibrous carriers. Process Biochem. 30, 661–665.

Macaskie, L.E., Wates, J.M., Dean, A.C.R., 1987. Cadmium accumulation by a *Citrobacter* sp immobilized on gel and solid supports; applicability to the treatment of liquid wastes containing heavy metal cations. Biotechnol. Bioeng. 30, 66–73.

Mozes, N., Rouxhet, P.G., 1984. Dehydrogenation of cortisol by *Arthrobater simplex* immobilized a supported monolayer. Enzyme Microb. Technol. 6, 497–502.

Ogbonna, J.C., Tomiyama, S., Liu, Y.C., Tanaka, H., 1997. Efficient production of ethanol by cells immobilized in loofa (Luffa cylindrical) sponge. J. Ferment. Bioeng. 84, 271–274.

Saudagar, P.S., Shaligram, N.S., Singhal, R.S., 2008. Immobilization of *Streptomyces clavuligerus* on loofah sponge for the production of clavulanic acid. Bioresource Technol. 99, 2250–2253.

Shukla, R., Chand, S., Srivastava, A.K., 2005. Improvement of gibberellic acid production using a model based fed-batch cultivation of *Gibberella fujikuori*. Process Biochem. 40, 2045–2050.

Shukla, R., Srivastava, A.K., Chand, S., 2003. Bioprocess strategies and recovery processes in gibberellic acid fermentation. Biotechnol. Bioproc. Eng. 8, 269–278.

Southgate, D.A.T., 1976. Determination of Food Carbohydrates. Appl. Sci. Publ. LTD, London. pp. 99–144.

Spatz, C., 1993. Basic Statistics, fifth ed. Brooks/Cole Puble. Co., California, Inc., USA .

Stanbury, P., Whitaker, A., Hall, S., 1995. Fermentation economics. In: Principles of Fermentation Technology, Second ed. Pergamann Press, Oxford, UK, pp. 331–341.

Tudzynski, B., 1991. Biosynthesis of gibberellin in *Gibberlla fujikuroi* bimolecular aspects. Appl. Microbiol. Biotechnol. 52, 298–310.

Vignoli, J.A., Celligoi, M.A.P.C., Silva, R.S.F., 2006. Development of a statistical model for sorbitol production by free and immobilized *Zymomonas mobilis* in loofa sponge *Luffa cylindrica*. Process Biochem. 41, 240–243.

Yellore, V., Desai, A., 1998. Production of poly-3-hydroxybutyrate from lactose and whey by *Methylobacterium* sp ZP24. Lett. Appl. Microbiol. 26, 391–394.