Mycoscience (2009) 50:323–330
DOI 10.1007/s10267-009-0488-y

FULL PAPER

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Acid phosphatase and invertase activities of *Aspergillus niger*

Received: June 13, 2008 / Accepted: January 29, 2009

Abstract *Aspergillus niger* is widely used as an enzyme source in industries. Considering its enzymic potential, *A. niger* was studied for its acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase), and invertase (EC 3.2.1.26, β-fructofuranoside fructohydrolase) activity in defined media supplemented with 1%, 3%, or 5% sucrose concentrations. Both these enzymes play a key role in phosphate and carbon metabolism in plants, animals, and microorganisms and hence are interesting from the standpoint of biotechnological applications. Ontogenic changes in extracellular, cytoplasmic, and wall-bound enzyme activities of *A. niger* were studied. Growth in terms of fresh weight showed inverse correlation with pH. At higher pH values, both enzyme activities were higher in the medium supplemented with low sucrose concentration. It was observed that the more the fresh weight of fungi decreased, the greater was the enzyme activity observed. It is suggested that these enzymes may participate in autolysis of fungi and, on the other hand, could prove to be a potential source of industrial application and exploitation.

Key words Acid phosphatase · *Aspergillus niger* · Invertase

Introduction

The filamentous fungi are a heterogeneous group of microorganisms that can use a wide variety of carbon and nitrogen sources for growth, exhibit great metabolic diversity, and furthermore can produce a wide range of secondary metabolites and hydrolytic enzymes, some of which are widely used in the food, beverage, and pharmaceutical industries (Metwally 1998). They produce extracellular enzymes to degrade insoluble substrate into smaller fragments and finally into soluble units that are then absorbed by hyphae (Chaube and Singh 2000). The property of microorganisms to produce enzymes is vastly exploited for industrial purposes.

Aspergillus niger, a cosmopolitan, widely distributed fungus, is an important industrial source for citric acid and enzymes, such as amylase, and enjoys a GRAS ("generally regarded as safe") status (Martens-Uzunova et al. 2006). The starch-degrading enzyme amyloglucosidase, which is an extracellular inducible enzyme from filamentous fungi (*Aspergilli*), is the second most widely produced enzyme worldwide after that of *Bacillus* protease (Metwally 1998). Thus, study of the stability of enzymes is an important aspect to consider in biotechnological processes as this can provide information on the structure of the enzymes and facilitate an economical design of continuous processes in bioreactors (Jurado et al. 2004).

Acid phosphatases (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) are a group of enzymes that catalyze the hydrolysis of external phosphate esters (Joh et al. 1996). They play an important role in the mineralization of organic carbon, organic phosphate, and low levels of free inorganic ions (Pi) (Straker and Mitchell 1986). Orthophosphate anion ("inorganic phosphate" Pi) plays a vital functional role in energy transfer and metabolic regulation and is also an important structural constituent of many biomolecules (Ehsanpour and Amini 2003). Extracellular acid phosphatases hydrolyze external phosphate esters, and intracellular acid phosphatases are involved in the routine utilization of Pi. Acid phosphatases of *Saccharomyces cerevisiae* have been extensively studied (Schweingruber et al. 1992). They have also been studied in *Aspergillus nidulans* (Arst et al. 1980), ectomycorrhizal fungi (Straker and Mitchell 1986), lysing and growing fungal structure, and molds (Haas et al. 1992). Acid phosphatase is reported to be located in the cell wall and septa of hyphae as determined by cytochemical studies by transmission electron microscopy (TEM) (Gutiérrez-Miceli et al. 2005).

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Invertases (EC 3.2.1.26, β-fructofuranoside fructohydrolase), a group of ubiquitous enzymes with different pH optima and subcellular localization (Verma and Dubey 2001), hydrolyze sucrose to glucose and fructose (Heil et al. 2005). It has been shown that sucrose must be hydrolyzed by cell wall invertase before entering the cell (Crasnier et al. 1980). The activity of invertase has often been investigated in diseased plants (Krishnan and Pueppke 1988). Considerable attention has been paid to the carbohydrate physiology of mycorrhizae, as the carbohydrate supply of the fungus is pivotal for the functioning of this symbiosis (Schaeffer et al. 1995). Nonphotosynthetic tissues prefer sucrose as a main carbon source, probably because of its high solubility, low reactivity, and energy storage capacity (Giaquinta 1980), where Pi plays a decisive role in multiple biosynthetic pathways (Chávez-Bárcenas et al. 2000). The intracellular phosphatases play an important role in the metabolism of carbohydrates (Giaquinta 1980).

Because fungi are opportunistic organisms, we have used sucrose with defined media for the study of acid phosphatase and invertase activity for their potential extraction. To the best of our knowledge, no such study has been conducted in *A. niger*. Thus, considering the importance of culture medium for growth and enzyme production, the present study aimed to investigate the extracellular, intracellular, and cell wall-bound acid phosphatase and invertase activities of *A. niger* with the course of time and at different levels of sucrose as a sole carbon source of the culture medium.

Materials and methods

Fungal strain and preparation of inoculum

Aspergillus niger (VP-001) strain was the same as that used in our previous work (Pawar and Thaker 2006). The inoculum was prepared according to Pawar and Thaker (2006), whereas the spore count was adjusted to 3.2×10^4 spores/ml.

Media preparation and culture conditions

Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (PT0018; HiMedia Laboratories, Mumbai, India) was used for the study. As the carbon sources, various concentrations of sucrose [i.e., 1% (A), 3% (B), and 5% (C)] were supplemented into the medium. The pH of the individual basal medium was adjusted to 5.5, of which 50 ml was placed in each 250-ml conical flask. The medium was autoclaved for 15 min at 121°C and allowed to cool to room temperature (RT). The inoculum (0.1 ml) was added to each flask and maintained in a stationary condition at 28°C.

Determination of hyphae diameter

Diameter was measured according to Pawar and Thaker (2006).

Extracellular, cytoplasmic, and wall-bound enzyme extraction

After 24 h, the culture media were collected from individual flasks under aseptic conditions every 12 h and filtered through a disk of Whatman no. 1 filter paper. This filtrate was used as the extracellular fraction for further assays. The mycelium that remained after removing the filtrate was gently blotted over the layers of filter paper and weighed. The mycelial mass was homogenized in a chilled pestle and mortar with acid-washed sand in sodium acetate buffer (100 mM, pH 5). This homogenate was centrifuged at 4°C at 8000 *g* for 30 min. The supernatant was used as the cytoplasmic fraction, and the residue was washed several times with repeated centrifugation by distilled water. It was then incubated in 1 M NaCl at RT (30° ± 2°C) overnight to release wall-bound fraction. Preliminary studies showed that 1 M NaCl was the optimal concentration for the release of wall-bound protein (Straker 1986). Three samples of each culture media were taken each time.

Acid phosphatase assay

The assay medium consisted of 0.3 ml 100 mM sodium acetate buffer (pH 5), 0.4 ml enzyme solution, and 2.3 mM *p*-nitrophenyl phosphate (pNPP) (in buffer) in a final volume of 1 ml. The mixture was incubated at RT (30° ± 2°C); after 15 min, incubation was terminated by the addition of 2 ml 1 M Na_2CO_3. In the control reaction, Na_2CO_3 was added before addition of enzyme. The absorbance of the yellow *p*-nitrophenyl (pNP) released was measured at 405 nm using an ELISA Reader (μ Quant; BioTek, Winooski, VT, USA). The quantity of pNP released was calculated from the calibration curve prepared using a range of pNP concentration from 0.036 to 0.7 mM prepared in the same buffer. The acid phosphatase activity was expressed as mM pNP released/gfwt/min for all three fractions (gfwt, grams fresh weight).

Invertase assay

The reaction mixture contained 0.15 M sucrose and enzyme solution. After 60 min incubation at RT (30° ± 2°C), the reducing sugars were estimated with the 3,5-dinitrosalicylic acid method (Miller 1959). Control was prepared by addition of Miller's reagent before the substrate. Absorbance was measured at 520 nm using an ELISA Reader (μ Quant, BioTek). Activity was calculated from the standard curve prepared by using glucose and expressed as milligrams (mg) sugar released/gfwt/min for all three fractions.

Protein determination

Total protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

Sugar estimation

The total sugar and reducing sugar were estimated from extracellular and cytoplasmic fractions. These fractions were mixed with 80% ethanol and incubated overnight. They were heated in a boiling water bath until the alcohol evaporated completely. The volume (10 ml) was made up with distilled water, and the extract was used for sugar estimation (both total and reducing) by the method of Thaker et al. (1992).

Statistical analysis

The mean values were statistically analyzed by analysis of variance (one-way ANOVA) using Tukey's test at $P \leq 0.05$ level of significance to detect the significant differences between media containing different sucrose concentrations: A (1%), B (3%), and C (5%).

Results and discussion

Murashige and Skoog's medium used in the present study is a defined medium, and known concentrations of sucrose were added to it to study their influence in enzyme production at different growth stages. There are few reports on the growth of molds considering the type of media (Bhaskaran and Smith 1993; Meletiadis et al. 2001). Moreover, even in the susceptibility tests the nutrient medium is a major factor that influences the results (Espinel-Ingroff et al. 1998). Sucrose is the major transport form for photoassimilates in higher plants; it is highly soluble, cheap, and is the major carbon source in molasses and an attractive feedstock for large-scale fermentation (Skowronek and Fiedurek 2006). As it is well known that sucrose is the common carbon source that is translocated in the plant system and is commonly used in tissue culture media, it was selected as a carbon source to be used in the present study. It is the principal carbohydrate substrate for the synthesis of cytoplasmic and cell wall constituents (Thaker et al. 1992). It has also been established that cAMP alteration of growth rate of *Aspergillus fumigatus* and *A. niger* is carbon source dependent (Oliver et al. 2002).

The diameter in the cellulose-hyperproducing mutant strain of *Penicillium* has been reported (Brown et al. 1987). In the present study, measurements of hyphal diameter showed increase with time in a sigmoid pattern (Fig. 1). With time the diameter increased and in later hours it was stabilized. Earlier hyphal diameter measurements were done in the inhibition studies of essential oils (Pawar and Thaker 2006), where diameter was less in tests than in the control.

Extracellular enzyme production in filamentous fungi is tightly coupled to hyphal tip growth and the increase in number of hyphal branches, which can be envisaged as increase in fresh weight (Fig. 2). The yield of mycelium in A (1% sucrose) reached a maximum (730 mg/flask) at 60 h and after that it declined, whereas that of mycelium in B

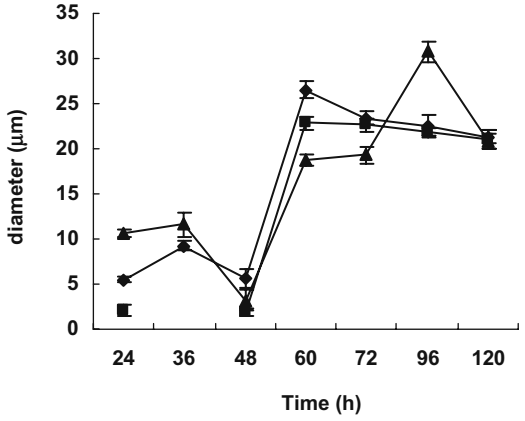

Fig. 1. Diameter of *Aspergillus niger* measured microscopically at different hours in media with 1% sucrose level A (◆), 3% sucrose level B (▲), and 5% sucrose level C (■) of sucrose concentration. *Vertical bars* represent ± standard deviation

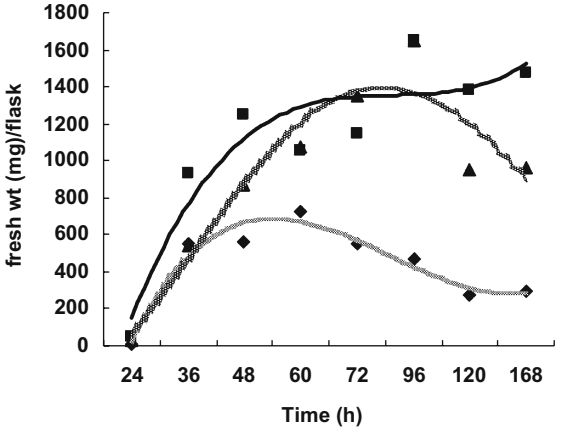

Fig. 2. Growth determination of *Aspergillus niger* grown in A (◆), B (▲), and C (■) in terms of fresh weight at different time periods. Values are statistically significant (at $P \leq 0.05$) between A and C. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

(3% sucrose) and C (5% sucrose) reached maximum (1650 mg/flask) at 96 h (Fig. 2). In the initial phase, up to 60 h, the growth was directly proportional to the sucrose levels. In A the fresh weight was lowest, followed by B and C, respectively. After 60 h, the growth of fungi moved parallel in B and C, but later on it declined in B and was stabilized in C. There was a statistically significant difference of A with B and C ($P \leq 0.01$), but this was not found between B and C.

Fungi as a group tend to be acidophilic with an optimum in the acid range. The pH of culture fluid with 1% sucrose (A) increased abruptly to 7.36 after 36 h until 60 h and remained constant thereafter. In B it remained between 6 and 3, but in C the pH sharply decreased and turned acidic from 72 h onward (Fig. 3). Thus, inverse correlation was observed among pH and growth of the fungi. Changes in pH with different levels of glucose have been studied showing that in low concentration of glucose pH was high and in high concentration of glucose pH was low (Maldonado and Strasser de Saad 1998). pH and fresh weight of

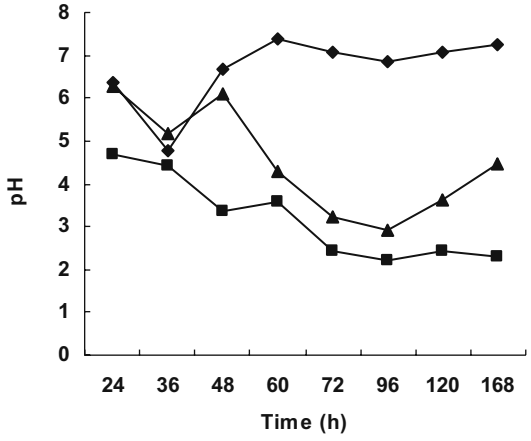

Fig. 3. Changes in pH with time in A (◆), B (▲), and C (■). Values are statistically significant (at $P \leq 0.05$) between A with B and C. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

the fungal mycelia do change according to the change in sucrose concentration. Thus, carbon source highly influences the growth, and in higher concentration the growth is maximum. It has been revealed that natural autolysis of *A. niger* occurs at pH 6.7 (Perez-Leblic et al. 1982). Thus, the decreasing fresh weight of the fungus in A might result from changing the pH toward alkalinity.

In the acid phosphatase assay, pNPP is used as a substrate that was shown to be most efficiently hydrolyzed in previous studies (Straker and Mitchell 1986). It has been reported that the catalytic efficiency of the enzyme given by the V_{max}:K_m ratio is much higher for the synthetic substrate (pNPP) than for the natural substrates (Gonnety et al. 2006). Acid phosphatase has been studied in yeasts and speculated to be associated with cell elongation (Miyata and Miyata 1978). The phosphatases are located on the surface of the yeast cell, which may indicate the possibility of adaptation to the outer nutritional environment. Their role in membrane processes costs an intriguing new light on the fact that these enzymes are periplasmic and extracellular in many microorganisms including *A. nidulans* (Arst et al. 1980).

In the present study, acid phosphatase activity was highest at 72 h in the extracellular fraction of A (Fig. 4a). In C, the activity remained consistently low during the entire time course, but in B the peak was observed at 24 and 120 h, i.e., in the initial and later phases of growth (Fig. 4a). The enzyme activity in A was significantly higher than in B and C. In the cytoplasmic fraction, at 24 h, maximum enzyme activity was observed in A, B, and C. Similar to the extracellular fraction, activity in cytoplasmic fraction of A was maximum, followed by B and C. In later hours, the activity peaked at 72 h in A and at 60 h in B and C; thereafter, it declined (Fig. 4b). In the wall-bound fraction also the activity declined after 48 h but at 72 h it showed a peak in A (Fig. 4c). Thus, the highest enzyme production was obtained in the initial phase at 24 h in the cytoplasmic fraction of A, B, and C. Media supplemented with the low sucrose concentration exhibited highest enzyme activity.

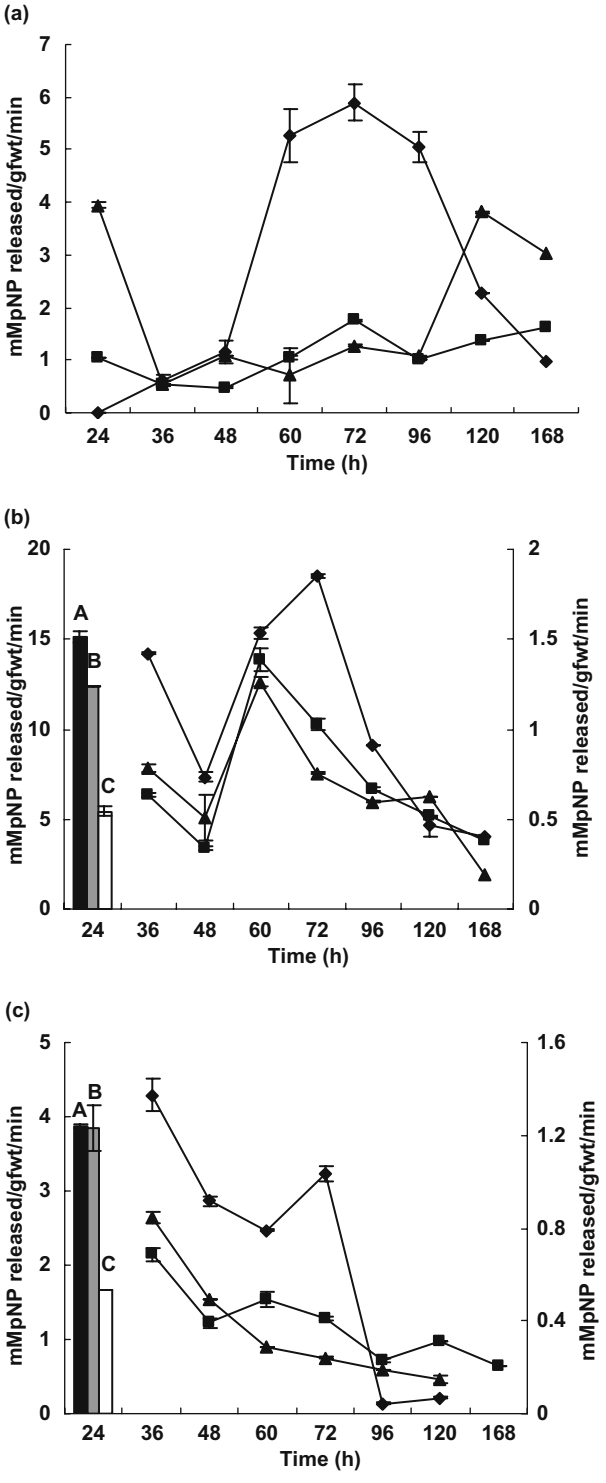

Fig. 4. Acid phosphatase activity (*p*-nitrophenyl, pNP) expressed on fresh weight basis (grams fresh weight, gfwt) in A (◆), B (▲), and C (■). **a** Extracellular; **b** cytoplasmic; **c** wall bound; the primary axis (*y*-axis) at *left* in **b** and **c** represents the bars. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

Extracellular phosphatase in the endophyte of *Erica hispidula* was more active than the cytoplasmic and wall-bound enzymes 18 days after inoculation (Straker and Mitchell 1986). Acid phosphatases have been reported in *Pholiota*

nameko (Yazaki et al. 1997), several ectomycorrhizal fungi, and VAM fungi (Dodd et al. 1987), suggesting their potential for degradation of complex phosphorus-containing compounds (Dodd et al. 1987) and their association with the growth and development of the fungus within the host tissue (Gianinazzi et al. 1979). With the bulk of the phosphate being organically bound, the phosphatases secreted by microorganisms would be important in the catalytic release of Pi from bound complexes (Straker and Mitchell 1986).

The increase in extracellular and wall-bound invertase activity was detected only during the later period of mycelial growth (Fig. 5a, c) but in the extracellular fraction at 24 h, the activity was found to be at the highest levels in A (25.57), followed by C (18.67) and B (10.3). The cytoplasmic invertase activity was also highest at 24 h in A (3.201) followed by B (1.058) and C (1.005); later on, it progressively increased at 60 h, and thereafter a sharp decrease in activity occurred (Fig. 5b). Thus, the highest enzyme production was obtained in the initial phase at 24 h in cytoplasmic as well as extracellular fractions of A, B, and C. The media supplemented with low concentration of sucrose exhibited the highest invertase activity.

The extracellular and cytoplasmic total sugars also peaked at 24 h in A, followed by B and C, respectively (Fig. 6a, b). In the later phase of growth total sugars were highest in both fractions of A. In contrast, they were lowest in B and C during this period, which may be attributed to their maximum weight of mycelia during the later phase compared to the low weight of mycelia in A. The sugars that are produced may be utilized in increasing the biomass in B and C.

The extracellular reducing sugar levels in A, B, and C were also maximum at 24 h, but here the value was highest in C (853.84), followed by B (276) and A (195), respectively, but decreased gradually with an increase in time period in all three media (Fig. 7a). The influence of different concentration of sugar is evident here. On the other hand, the cytoplasmic reducing sugar levels, which were also found maximum at 24 h, peaked in A (196.27), followed by C (20.1) and B (16.88), respectively (Fig. 7b). In the later hours it peaked at 60 h in C, 168 h in B, and at 120 h in A. Highest reducing sugars at 24 h correlate with maximum invertase activity at the same time. Cytoplasmic reducing sugars were high at 60 h in C with maximum invertase activity. Invertase activity after 72 h gradually declined in all (Figs. 5b, 7b).

In *Saccharomyces cerevisiae*, the utilization of sucrose involves extracellular hydrolysis followed by transport of the liberated hexoses into the cell (Sutton and Lampen 1962). Thus the total conversion of sucrose to reducing sugars and its decline over time supports this conclusion (Bhaskaran and Smith 1993). Extracellular invertase activity was low at 36 h and 48 h in A and B; it reached maximum at 120 h. However, the reducing sugars were high at initial hours and decreased later on. Thus, as the invertase activity is increasing, more reducing sugars are accumulated in the cytoplasmic fraction, and less sugars are found in the extracellular fractions in the later phase. Production of more reducing sugars in the later phase may indicate the invertase activity is increasing.

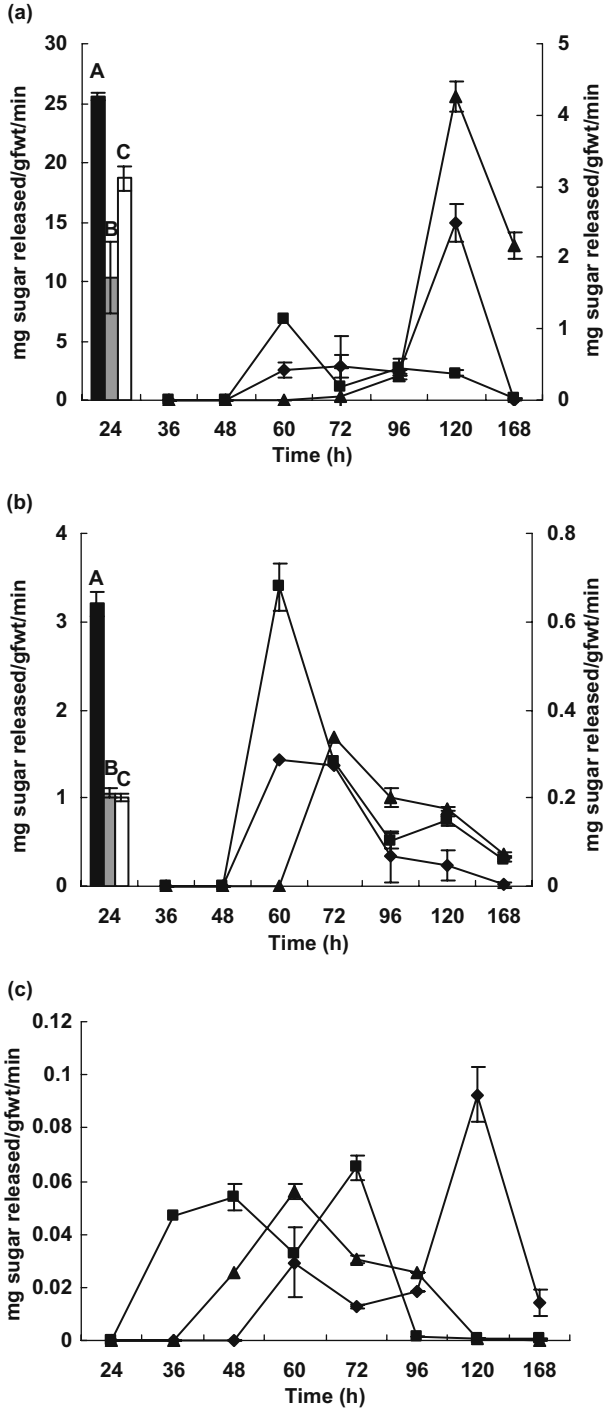

Fig. 5. Invertase activity expressed on fresh weight basis in A (◆), B (▲), and C (■). **a** Extracellular; **b** cytoplasmic; **c** wall bound; the primary axis (*y*-axis) at *left* in **a** and **b** represents the bars. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

In previous studies it has been speculated that *Sporisorium relianum* is potentially capable of producing extracellular enzymes for the release of hexoses (glucose and fructose) into the medium, which are used for growth in the yeast form (Bhaskaran and Smith 1993). By using the

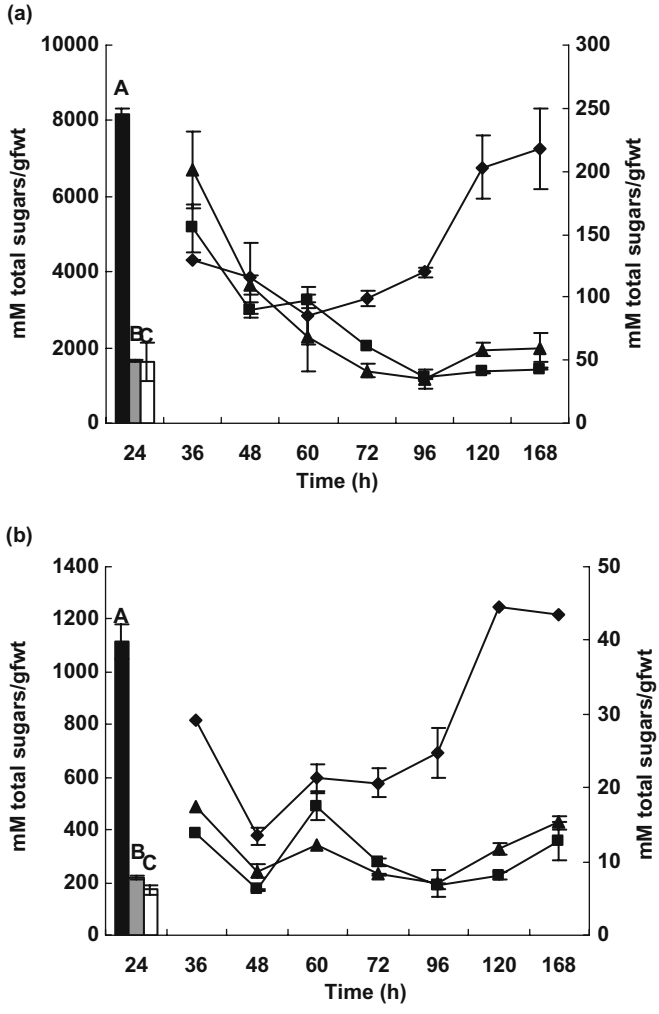

Fig. 6. Changes in total sugars in A (◆), B (▲), and C (■) with time. **a** Extracellular; **b** cytoplasmic; the primary axis (*y*-axis) at *left* represents the bars. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

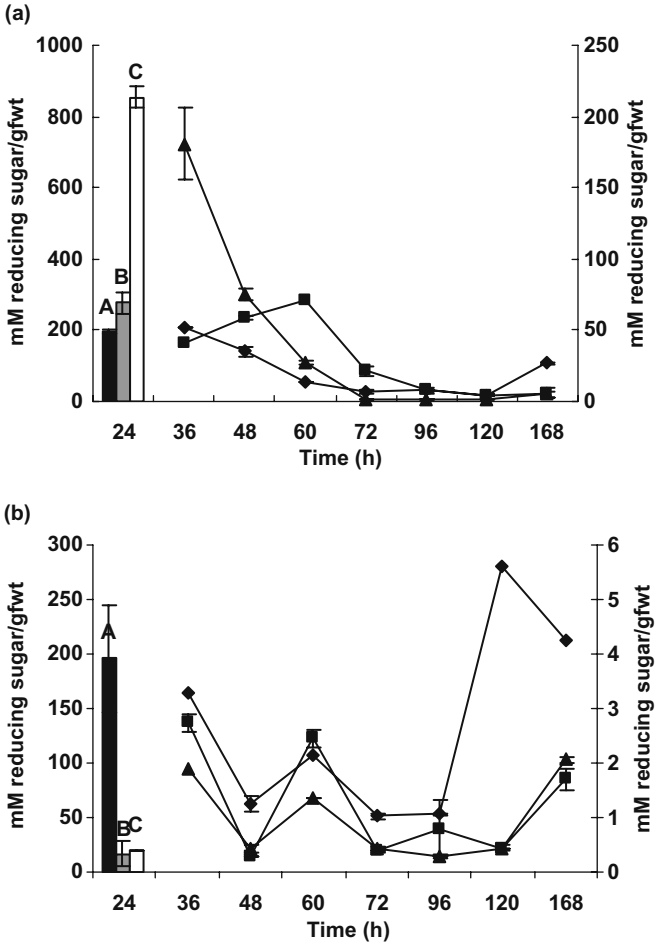

Fig. 7. Changes in reducing sugars in A (◆), B (▲), and C (■) with time. **a** Extracellular; **b** cytoplasmic; the primary axis (*y*-axis) at *left* represents the bars. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

defined carbohydrates, they showed that invertase is produced by the fungus (Bhaskaran and Smith 1993). Wall-bound invertase activity was also found to be highest at 60 to 120 h, which proves that maximum uptake of sugars takes place during the later phase to facilitate the growth of fungi. Invertase activity is also higher in A than in B and C. Thus it is possible that to thrive in the alkaline condition and to increase growth in the later phase, the fungus expresses more enzyme activity in 1% sucrose than in 3% and 5% sucrose.

Invertase and acid phosphatases have been studied in several host–pathogen interaction studies. A reduction in phosphatase activity was shown in tobacco leaves after invasion with *Pholiota tobacina* in the necrotic phase of the development of the disease. This decrease, as it had become clear from the cytochemical investigations, affects the tissues of the host, whereas the hyphae of the parasite have highly active phosphatase (Edreva and Georgieva 1977). Alternatively, the acid phosphatases may play a role in recognition and/or infection processes, as has already been suggested for such enzymes in other biological systems. Reviewing nutrient relationships in biotrophic infections emphasized that acid invertase is the most studied relevant enzyme in infected leaves, and it is of central interest in view of its probable role in hydrolysis of sucrose before sugar uptake by fungi (Schaeffer et al. 1995). The acid invertase activity was inversely proportional to the starch content and closely related to the changes in saccharose and glucose content (Pius et al. 1998). The significantly enhanced enzyme activity began with the onset of sporulation and reached a maximum at the stage of maximum basidiospore release, when it was nearly ten times higher than in the control (Pius et al. 1998). Although the localization of invertase is not yet clear (Scholes et al. 1994), its increasing activity during sporulation may be caused by a fungal enzyme as previously proposed (Xiu et al. 1993).

Evaluation of protein in extracellular fraction of A, B, and C showed that at the initial phase, protein was greatest in A (1439 mg/gfwt), followed by B (911 mg/gfwt) and then C (674 mg/gfwt), respectively, but at 72 h the protein value was greater in the medium with a greater sucrose concentration (Fig. 8a). Cytoplasmic protein value was greater in C

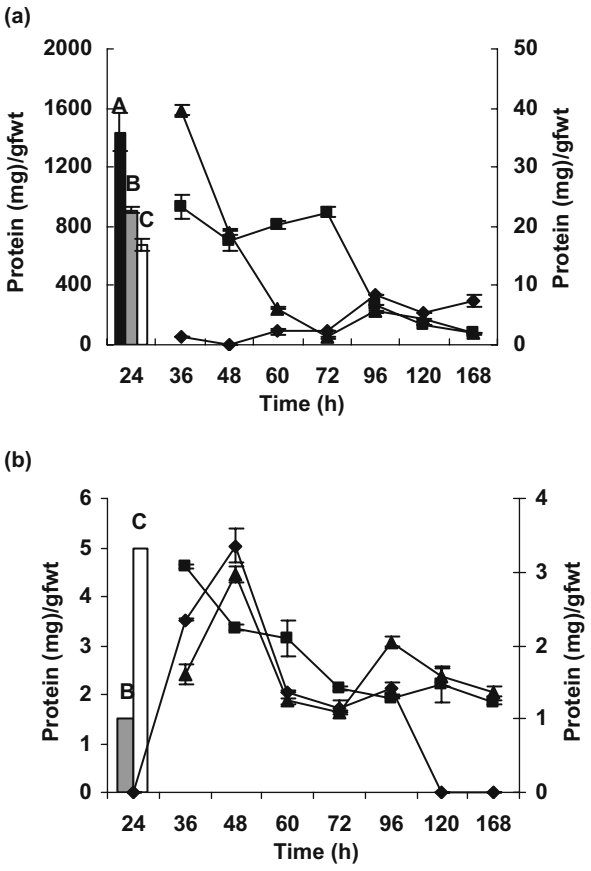

Fig. 8. Protein content changes A (◆), B (▲), and C (■) with time. **a** Extracellular; **b** cytoplasmic; the primary axis (*y*-axis) at *left* represents the bars. Symbols A–C refer to sucrose levels (see legend, Fig. 1)

(4.98 mg/gfwt) at 24 h and 36 h; after that, it gradually declined. However, the protein value in A and B peaked at 48 h and then declined (Fig. 8b).

In the present study, enzyme activity expressed in A is several fold higher compared to B and C in the early and later growth phases. As the pH changes toward alkalinity, the weight of the fungus decreases (see Figs. 2, 3). Thus, with a low concentration of sucrose, although biomass is low, enzyme activity is enhanced. Furthermore, high enzyme activity could be partly the result of increased pH; again, this idea needs further experimentation. However, it might be suggested that alkaline pH could be stressful for growth and development and so to thrive in such conditions the fungi might have produced more enzyme to fulfill its nutritional requirements. Furthermore, it was previously reported that during the autolytic condition at higher pH, generally the lytic enzymes that attack the cell wall and the reserve carbohydrates are activated (Gomez et al. 1997). This report supports that the decrease in weight might be caused by autolysis at alkaline pH. Moreover, in culture medium with 3% and 5% sucrose concentration, enzyme activities remained low in comparison to culture medium with 1% sucrose concentration. With a suitable carbon source, pH, and optimal concentration, fungi in B and C grow well with more protein value and with minimum need to increase acid phosphatase and invertase activities, i.e., the enzymes involved in phosphate and carbohydrate metabolism.

Thus, the present work showed that concentration of sucrose influences fungal growth, pH, and enzyme production. These enzymes not only can be used to understand the host–pathogen relationship but also can be utilized in industries for various applications. By regulating sucrose concentration and pH continuously, mass production of these enzymes may be successful.

Acknowledgments The authors are thankful to Vimal Research Society for Agro-Biotech and Cosmic Powers for providing necessary laboratory facilities. The first author is also thankful to Department of Biosciences (Special Assistance Programme – SAP) and Gujarat State Biotechnology Mission (GSBTM) for providing financial support.

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