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Proteomic Response to Intracellular Proteins of *Monascus pilosus* Grown under Phosphate-Limited Complex Medium with Different Growth Rates and Pigment Production

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Monascus pigments are important colorings in food applications. Rice containing potassium phosphate and sodium nitrate was reported as a good pigment-producing medium for *Monascus* in previous studies. We found that the lack of potassium phosphate in this medium depressed red pigment production in cultivated *Monascus pilosus*. However, the influence of phosphate limitation on the biochemical metabolisms concerning culture growth and pigment production in *Monascus* remains unknown. Here, we used proteomic analysis by two-dimensional gel electrophoresis, matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), tandem mass spectrometry (MS/MS), and database interrogation to separate and identify the proteins of *M. pilosus* grown between the lack of potassium phosphate and the control media. Phosphate limitation to this complex medium induced an up-regulation of aldehyde dehydrogenase and several glycolytic enzymes in *Monascus* relative to the control. In contrast, the metabolic enzymes such as glucosamine:fructose-6-phosphate aminotransferase and ADP-ribosylation factor 1 were up-regulated in the control.

KEYWORDS: ***Monascus pilosus*; food pigments; protein extraction; proteomics**

INTRODUCTION

It is well-known that *Monascus* pigments have been used as natural colorants and traditional food additives in Asia for centuries. Since the findings that coal tar dyes are carcinogenic, the natural pigments produced by *Monascus* have been considered as a safe natural food color and replacement for synthetic pigments (*1*, *2*). It has been known that *Monascus* can produce at least six major related pigments, divided into three groups (*3*, *4*). To date, there are many reports in previous studies and our studies concerning growth media and optimal cultivation conditions applied to various forms of fermentation to increase pigment and metabolites production in *Monascus* (*5*−*10*). Despite research efforts that spanned decades, how most growth substrates affect *Monascus* cellular mechanisms remains unknown. Rice is considered as a good carbon source for cultivation of *Monascus*. Tseng et al. reported a good pigment-producing medium in *Monascus*, containing rice powder, $NaNO_3$, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4 (*11*, *12*). In our work, we found the lack of KH_2PO_4 in this medium induced a markedly different growth pattern and pigmentation in cultivated *M. pilosus*. Phosphate is essential for all living organisms as a structural component of nucleic acids and phospholipids, a constituent of energy transfer reactions, and a regulator in signal transduction cascades. However, little is known about the influence of phosphate limitation in growth medium on the cellular metabolisms concerning culture growth and pigment production in *Monascus* during fermentation.

On the basis of the high resolution of two-dimensional electrophoresis (2-DE) and mass spectrometry, proteomics is a powerful tool for analysis of hundreds of proteins expressed in a complex mixture at one time as different samples of plant cells (*13*), yeast cells (*14*), and bacteria cells (*15*, *16*). The proteome provides a better understanding of dynamic and overall views of the cell machinery under various conditions. However, compared to yeast, plant, and animal analyses, only very few studies on 2-DE of filamentous fungi are reported (*17*, *18*). Advances in mass spectrometric instrumentation have coincided with the availability of increasing amounts of genomic sequence data. Protein identification is now possible by means of peptide mass and fragmentation data generated by mass spectrometric analysis, matched against a database of all possible proteins encoded by a genome (*19*). When the genome sequence of the organism being studied is known, data obtained from MALDI-TOF MS can theoretically provide the identity of any unknown protein using peptide mass fingerprints (PMF). However, when little genomic sequence data are available for the organism, a different approach must be taken. Our approach is based on PMF and MS/MS of individual peptides, matched against

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genomic sequence database in related species with cross-species identification (CSI) (*20*, *21*), and has previously been shown to be successful in the direct identification of proteins from large genomes (*22*).

Filamentous fungi, such as *Aspergillus* and *Monascus*, have the unique ability to economically produce and secrete many different types of products (e.g., commodity chemicals, enzymes) and offer a number of benefits over other types of cells. Unfortunately, few filamentous fungal genomes are publicly available, making DNA microarray analysis difficult. Alternatively, a proteomics approach would be useful for functional genomics studies. Proteomics in filamentous fungi is still at a relatively early stage of development, particularly of intracellular proteins. Recently, the genome of *Monascus* has been sequenced (*Monascus* genome database). Much effort will be dedicated to generate comprehensive information about the function of the genes in the *Monascus*'s genome. However, currently, only about 42 entries of *Monascus* protein sequences are in the SwissProt/TrEMBL database (http://www.expasy.org/sprot/). Cloning experiments are expensive and laborious. The unique characteristics of *Monascus* in the formation of secondary metabolites deserve our attention, and therefore, we conducted this proteomic study for identification and quantification of *Monascus* proteins. The objective of this work was to use proteomic analysis to investigate the influence of lack of phosphate substrate to this rice nitrate complex medium on the biochemical metabolisms concerning culture growth and pigment production in cultivated *Monascus*. A comparison of protein pattern expressions in two different cultivations of *M. pilosus* was performed using 2-DE, matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), and database searches to identify metabolic proteins. We show here that the phosphate limitation in the rice nitrate complex medium caused the inhibition of rice carbon source consumption in the medium and, in parallel, induced an up-regulated expression of aldehyde dehydrogenase and several glycolytic enzymes in the phosphate limitation-cultivated *Monascus*.

MATERIALS AND METHODS

Culture Strain and Cultivation Conditions. The strain of *Monascus pilosus* BCRC 31527 used for this study was obtained from the Bioresource Collection and Research Center (BCRC) in Taiwan. *Monascus* was sporulated and maintained on potato dextrose agar (Merck; Darmstadt, Germany) in stock culture. The control medium for pigment production was a rice sodium nitrate medium (R) consisting of 3% w/v *indica* rice powder (*Oryza sativa*, L. *indica*), 0.15% w/v $NaNO_3$, 0.1% w/v $MgSO_4 \cdot 7H_2O$, and 0.25% w/v KH_2PO_4 according to the method of Tseng et al. (*11*, *12*). Modified media for experiments were R without $NaNO_3$ (MK), R without $MgSO_4 \cdot 7H_2O$ (NK), and R without KH_2PO_4 (NM), respectively. The *indica* rice powder, containing 76.3% w/w carbohydrate, 8.2% w/w protein, and 77 mg phosphorus per 100 g of rice powder, was purchased from Sunlight (a local rice powder producer; Taipei, Taiwan). Accordingly, the carbon to nitrogen mass ratio in the R, NK, and NM media is 17.5:1 and the ratio in the MK medium is 28.5:1. In cultivation conditions, *Monascus* spores, prepared by growth on potato dextrose agar slants for 10 days at 32 °C, were washed with a sterile phosphate buffer (50 mM, pH 7.0). A suspension of 10^8 spores was used to inoculate a 1 L baffled Erlenmeyer flask containing 250 mL of R, MK, NK, and NM media (before sterilization, the pH of the media was adjusted to 6.2 with 1 N HCl or 1 N NaOH (*23*)), respectively, which were cultivated at 32 °C for 10 days for submerged fermentation on a rotary shaker at 150 rpm in triplicate.

Reagents and Materials. The two-dimensional electrophoresis reagents including acrylamide solution (25%), urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), dithiothreitol (DTT), Immobiline Dry Strips, immobilized pH gradients (IPG) buffer, IPG cover mineral oil, Tris base, sodium dodecyl sulfate (SDS), iodoacetamide (IAA), trifluoroacetic acid (TFA), and protein assay kit were purchased from Bio-Rad (Hercules, CA). Phenylmethanesulfonyl fluoride (PMSF) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma (St. Louis, MO). Sypro Ruby stain was purchased from Amersham Biosciences (Piscataway, NJ). Trypsin (modified) was obtained from Promega (Madison, WI). ZipTip C18 microcolumns were purchased from Millipore (Bedford, MA).

Estimation of Red Pigment and Residual Carbon Source Content. Estimation of red pigment was done according to the method of Tseng et al. (*11*, *23*). At the end of fermentation, the mycelia from each flask were filtered and washed twice, each time with 20 mL of distilled water. The washed mycelia were then extracted with 80 mL of 95% ethanol for 12 h. The filtered solution and extract were made to 100 mL, respectively, and the optical density at 500 nm was measured in a spectrophotometer (Thermo Helios α, Waltham, MA). The two values (extracellular pigment in the filtered solution plus cell-bound pigment in the extract) were added to give the total absorbance of pigment production. For dry cell mass, the mycelia after extraction were lyophilized in a vacuum-freeze dryer (EYELA FDU-540, Tokyo, Japan) for 2 days. After fermentation, the residual carbon source content of rice starch in the supernatant of the R and NM media was hydrolyzed into glucose by 2 N HCl solution. Concentrations of glucose derived from hydrolyzed residual rice starch powder in the supernatant of medium were determined by HPLC (Aminex HPX-87H ion column, Bio-Rad; temperature, 35 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.6 mL/min; detector, refractive index).

Sample Preparation for Two-Dimensional Electrophoresis. After centrifugation of the culture broth at 20000*g* and 4 °C, the cell pellets were washed twice with 50 mM phosphate buffer (pH 7.0) and stored at −70 °C for further analysis. Cells were extracted in a Tris-HCl buffer, containing 50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.5 mM PMSF, and 1 mM DTT using a blender for 5 min and were sonicated (sonicator XL 2000, Misonix, NY) on ice for 5 min. The cell suspension was disrupted using a continuous type presser (Constant Cell Disruption Systems Z plus, Z5/40/CB/GA, Constant Systems Ltd., Northants, England) at 30 kpsi, 4 °C, followed by centrifuging at 40000*g*, 4 °C, to obtain the supernatant. The resulting supernatant was then mixed with ice-cold TCA and DTT to a final 20% w/v and 0.2% w/v concentration, respectively. After incubation at −20 °C overnight, the sample was centrifuged at 7000*g* for 30 min at 4 °C. The pellets were washed with ice-cold acetone containing 0.1% w/v DTT at −20 °C for at least 30 min and then centrifuged again. Finally, the wash step used above was repeated without DTT and the precipitated protein was lyophilized.

Two-Dimensional Electrophoresis. The protein pellet was solubilized completely in a sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% ampholine, 65 mM DTT, with a trace of bromophenol blue) and left to stand for 1 h at 4 °C. After 10 min of centrifugation at 18000*g* at 4 °C, an amount of 350 μL of supernatant containing 300 μg of proteins was loaded onto the IPG strip. Protein content was determined by the Bradford method (protein assay kit, Bio-Rad). Isoelectric focusing (IEF) was carried out via stepwise voltage increments with the following conditions: step 1, rehydration, 10 V for 12 h; step 2, 100 V for 1 h; step 3, 250 V for 1 h; step 4, 500 V for 1 h; step 5, 1000 V for 1 h; step 6, 4000 V for 1 h; step 7, 8000 V for 45 000 V/h; total, 50 975 V/h. Following IEF separation, the gel strip was first equilibrated for 15 min in an equilibration buffer containing 50 mM Tris (pH 8.8), 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 2% w/v DTT and subsequently in the same buffer with 2.5% w/v IAA replacing DTT for another 15 min. The equilibrated strip was then transferred onto the second-dimensional SDS−PAGE gel and sealed in place with 0.5% agarose. SDS−PAGE was performed on 10−18% gradient polyacrylamide gel (18.5 cm × 18.5 cm × 1.5 mm) at a constant voltage of 10 mA for 0.25 h and 45 mA for 3.5 h until the dye front reached the bottom of the gel. Sypro Ruby stain was used for visualization of the 2-DE gel. The fluorescence-stained 2-DE gel was digitally scanned as a 2-DE image on a Typhoon 9200 fluorescence image scanner (Amersham Biosciences). Spot detection,

quantification, and matching were managed using PDQuest software (Bio-Rad). The theoretical molecular mass (M_r) and p*I* values of the 2-DE markers were used to calibrate the M_r and p*I* of the protein spots in the 2-DE gels. Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel, and the protein quantity of each spot was calculated by integrating the density over the spot area.

In-Gel Tryptic Digestion. The protein spots detected on 2-DE were manually excised from the gel and cut into pieces. The pieces were then washed twice with 50% v/v acetonitrile (ACN) in 25 mM ammonium bicarbonate, pH 8.5, for 15 min each time, dehydrated with 100% ACN for 5 min, dried, and rehydrated with a total of 100 ng of sequencing grade, modified trypsin in 25 mM ammonium bicarbonate, pH 8.5, at 37 °C for 16 h. Following digestion, tryptic peptides were extracted twice with 50% ACN containing 5% v/v formic acid for 15 min each time with moderate sonication. The extracted solutions were pooled and evaporated to dryness under a vacuum. Dry peptide samples were redissolved in 0.1% v/v TFA and purified by ZipTip C18 microcolumn according to the manufacturer's instruction manual, where necessary.

MALDI-TOF/TOF MS. MALDI-MS detection and MS/MS sequencing of isopeptides in reflection mode were performed on an Applied Biosystems 4700 proteomics analyzer mass spectrometer (Applied Biosystems, Framingham, MA) equipped with an Nd:YAG laser (355 nm wavelength, <500 ps pulse, and 200 Hz repetition rate in both MS and MS/MS modes). The 1000 and 2500 shots were accumulated in positive ion mode MS and MS/MS mode, respectively. The tryptic digested peptide samples were dissolved in 50% ACN with 0.1% formic acids and premixed with a 5 mg/mL matrix solution of CHCA in 70% ACN with 0.1% formic acid for spotting onto the target plate. For collision-induced dissociation (CID) MS/MS operation, the indicated collision cell pressure was increased from 3.0×10^{-8} Torr (no collision gas) to 5.0×10^{-7} Torr, with the potential difference between the source acceleration voltage and the collision cell set at 1 kV. The resolution of the timed ion selector for the precursor ion was set at 200. MS data were acquired using the instrument internal calibration. At a resolution above 10 000 in MS mode, accurate mass measurement (<50 ppm) of the monoisotopic isopeptide signals is possible. MS/MS data were acquired using the instrument default calibration.

Data Search. After data acquisition, the files were searched by querying the SwissPort and/or NCBI database using MASCOT (http://www.matrixscience.com) with the following parameters: peptide mass tolerance, 50 ppm; MS/MS ion mass tolerance, 0.25 Da; allowance of up to one missed cleavage; consideration of variable modifications that were methionine oxidation and cysteine carboxyamidomethylation; fungi and bacteria taxonomy category.

RESULTS

Different Growth Media for Pigment Production in *Monascus*. In this work the effect of different ingredients in this rice nitrate complex medium (R) (*11*, *12*) on the pigment productivity of *M. pilosus* BCRC 31527 was examined first, and then the influence of the lack of KH_2PO_4 in this R medium on the metabolism of this cultivated fungus was investigated using proteomic technology.

In our previous screening experiments, we evaluated the productivity of the red pigment of 11 *Monascus* strains on the R medium by submerged fermentation. It was found that *M. pilosus* BCRC 31527 revealed the maximum level of red pigment production among the 11 strains (data not shown). Here, by use of four media, i.e., R (the control), MK (R lacking $NaNO_3$), NK (R lacking $MgSO_4 \cdot 7H_2O$), and NM (R lacking KH_2PO_4) media, respectively, to cultivate *M. pilosus* BCRC 31527 in submerged fermentation for 10 days, the effect of the media on red pigment productivity revealed that the R and NK media were significantly greater than the NM and MK media at the seventh day of the cultivation (Scheffe's test, $p < 0.05$)

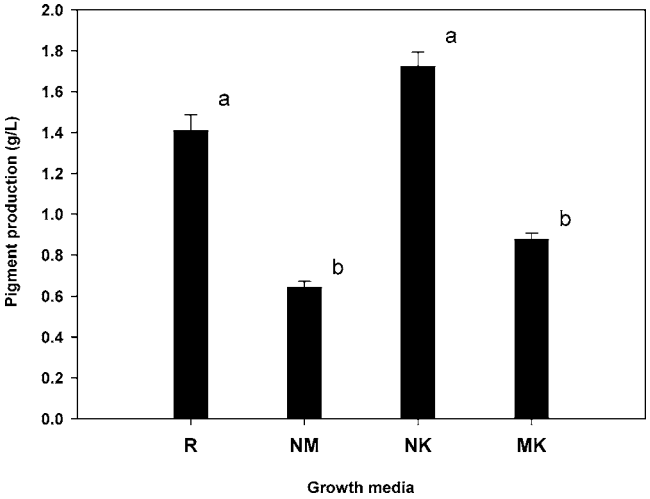

Figure 1. Effect of different growth media on the pigment production in *Monascus pilosus* BCRC 31527 at the seventh day in batch type of submerged fermentation on a rotary shaker at 32 °C and 150 rpm for 10 days of cultivation. R, NM, NK, and MK represent different media (see Materials and Methods). The data shown were the mean values ± SEM from three independent experiments. Letters a and b represent no significant differences while it has the same letter above each medium bar, Scheffe's test ($p < 0.05$).

(**Figure 1**). Moreover, when the strain cultivations between the control (R) and NM media during fermentation were compared, the cell mass and pigment production of the control were obviously higher than the corresponding values of the NM medium after the third day of fermentation (**Figure 2B**). In parallel, the carbon source content in the control was consumed more quickly than the corresponding value in the NM medium during cultivation (**Figure 2A**).

Proteomic Analysis of Intracellular Proteins. In an attempt to characterize the influence of phosphate limitation of the NM medium on the cellular metabolisms of the fungus during cultivation, cells were harvested for 2-DE analysis on the fifth day of cultivation, since that time point reflected the maximum of substrate-inducing differences in pigment production and cell mass between the control and the NM media. A comparative 2-DE Sypro Ruby-stained gel analysis of the cells grown under the control and the NM media, respectively, on the fifth day of cultivation is illustrated in **Figure 3**. The most pressing problem in 2-DE analysis is image registration, which ensures that identical proteins in different gels are recognized as being identical. With registration of several gel images, a single reference or consensus gel image that combines the information content of all individual images was generated. Here, this reference gel consisted of a representative set of spots generated from three registered gel images of the control cells or the cells of NM medium and was used to compare each sample. Analysis of spots on the 2-DE gel indicated that around 600 proteins had a molecular mass of less than 97 kDa and a p*I* within 3−10. Among them, the 76 spots that were determined with different expressions in protein content were cut and analyzed first using MALDI-TOF/TOF MS. By a search of the protein database against all fungal species through SwissProt and/or NCBI on MASCOT, four proteins (dihydroorotate dehydrogenase, peroxisomal NADP-dependent isocitrate dehydrogenase, nitrogenase iron protein, and pyruvate kinase (PK)) were identified. These peptide mass fingerprints (PMF) with cross-species identification (CSI) matched known proteins from the sources

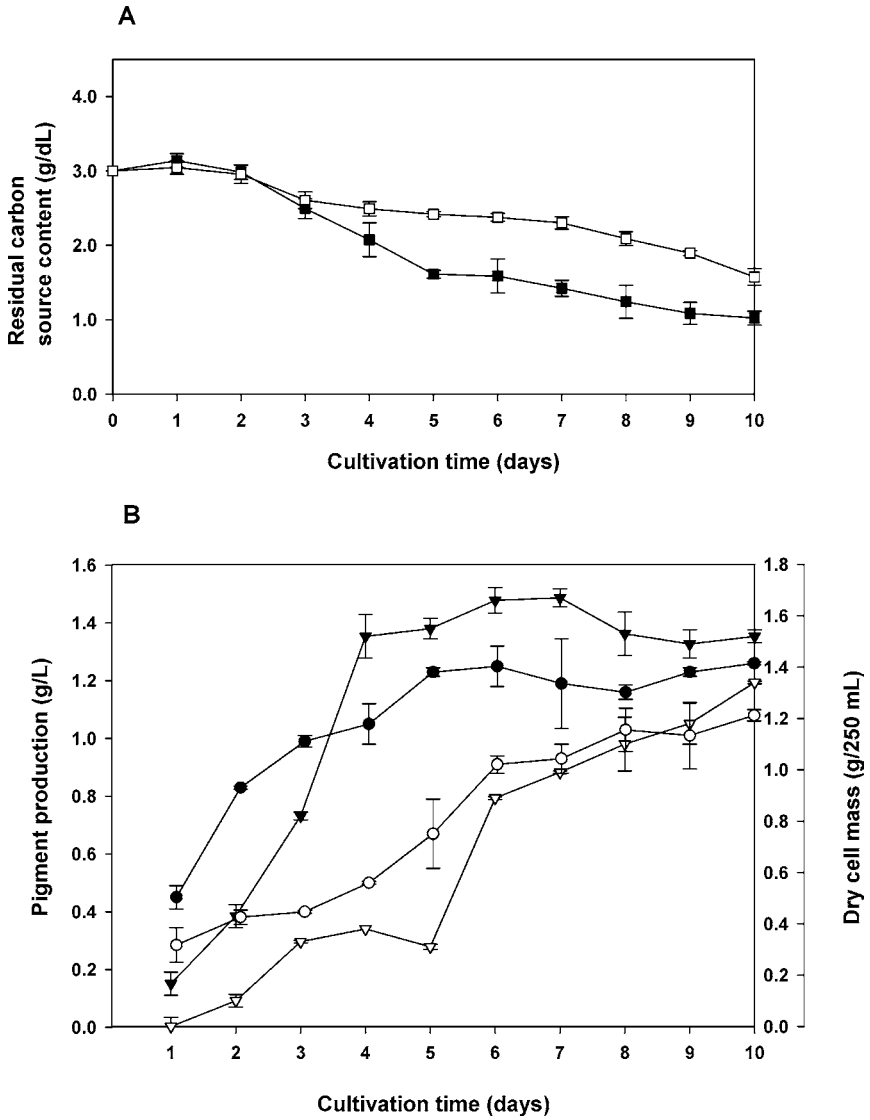

Figure 2. Culture development of *Monascus pilosus* BCRC 31527 in batch type of submerged fermentation on a rotary shaker at 32 °C and 150 rpm for 10 days: (●) dry cell mass of R medium; (▼) pigment production of R medium; (■) residual carbon source content in R medium; (○) dry cell mass of NM medium; (▽) pigment production of NM medium; (□) residual carbon source content in NM medium. R represents the rice nitrate complex medium, and NM represents R lacking KH_2PO_4 complex medium. The data shown were the mean values ± SEM from three independent experiments. In part **A**, the residual carbon source content in the medium was assayed by hydrolyzing rice starch into glucose, determining glucose level as residual carbon source content.

in *Saccharomyces paradoxus*, *Aspergillus nidulans*, *Azorhizobium caulinodans*, and *Yarrowia lipolytica*, respectively (**Table 1**) (*20*, *21*).

When those proteins were not able to be identified through the PMF, we subsequently used MALDI-TOF/TOF MS and MS/MS for protein identification. In these, seven protein spots were identified from the sources in the related species' genes by CSI, and another three proteins, including nucleoside diphosphate kinase, aldehyde dehydrogenase (ALDH), and alkaline proteinase, came from the sources in *A. nidulans*, *Aspergillus niger*, and *Aspergillus fumigatus*, respectively (**Table 1**). Comparison of the MS/MS analysis of amino acid sequences in the five PMF peptides on spot 13 yielded scores 93−220 of identity to enolase 1 in three fungal genuses (*Alternaria alternate*, *Davidiella tassiana*, and *A. niger*). Another enolase was also identified using MALDI-TOF/TOF MS and MS/MS, and two peptides could be matched to gi|37147852 from *Tuber borchii* in the NCBI database. In addition, protein spot 4 was identified as an ADP-ribosylation factor, which is a 21 kDa protein of the Ras superfamily of GTP-binding proteins, from *Giardia* to human, and which is greater than 60% identical (*24*−*26*).

Expression Profiling of Metabolic Enzymes in Cultivated *M. pilosus* BCRC 31527. A comparison of protein contents of the metabolic enzymes identified in this work is depicted in **Figure 4** by two independent 2-DE experiments and PDQuest program-aided analysis. Relative values of protein content were calculated by dividing each value by the highest protein content of enolase 1 on the 2-DE gel of the cells of NM medium. Interestingly, ALDH, adenylate kinase (AK), and several glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase 1, and PK, were up-regulated in the NM medium relative to the control. By comparison with the NM medium, in the control several other identified enzymes, including dihydroorotate dehydrogenase, formate dehydrogenase-like protein, peroxisomal NADP-dependent isocitrate dehydrogenase, and metabolic enzymes, such as glucosamine: fructose-6-phosphate aminotransferase (GFAT) and ADP-ribosylation factor 1, were induced to be up-regulated.

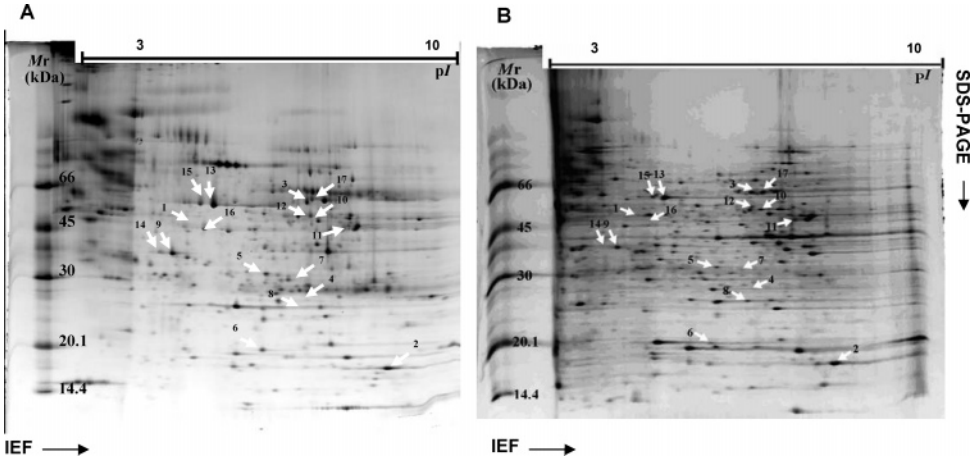

Figure 3. Comparative 2-DE gel analysis of *Monascus pilosus* BCRC 31527 cultivated between the R medium (**A**) and NM medium (**B**) for 5 days in submerged fermentation at 32 °C and 150 rpm. The protein extracts (300 μg/350 μL) of *M. pilosus* BCRC 31527 were displayed across a linear IPG strip (pH 3–10, 18 cm) in the first dimension and a 10–18% linear gradient SDS–PAGE in the second dimension, stained with Sypro Ruby. Isoelectric point (p*I*) and molecular mass (M_r) were marked on horizontal and vertical axes, respectively. Among the protein spots analyzed with the PDQuest program, 17 spots exhibiting different expression levels were indicated by white arrows and numbers. Details of the proteins are given in **Table 1**.

Table 1. List of Proteins Isolated from 2-DE Gel of *M. pilosus* BCRC 31527 and Identified by MALDI-TOF/TOF MS Analysis

spot no.	protein names	sources	M_r [a] (kDa)	p*I* [a]	M_r [b] (kDa)	p*I* [b]	sequence coverage (%)	score	accession number[c]	database	type of analysis[d]
1	dihydroorotate dehydrogenase	*Saccharomyces paradoxus*	35	5.9	45	5.3	20	24	Q7Z891	SwissProt	MS
2	nucleoside diphosphate kinase	*Emericella nidulans* (*Aspergillus nidulans*)	17	7.6	17	7.8	11	39	Q8TFN0	SwissProt	MS/MS
3	aldehyde dehydrogenase	*Aspergillus niger*	54	6.0	55	6.7	4	34	P41751	SwissProt	MS/MS
4	ADP-ribosylation factor 1	*Saccharomyces cerevisiae*	20	5.5	21	7.0	22	26	P07170	SwissProt	MS/MS
5	formate dehydrogenase-like protein	*Magnaporthe grisea*	40	6.0	32	6.2	20	45	gi|58257473	NCBI	MS/MS
6	D-arabino-3-hexulose 6-phosphate formaldehyde lyase	*Pyrobaculum aerophilum* str. IM2	19	5.5	23	6.1	39	36	gi|18312803	NCBI	MS/MS
7	glycoprotein endopeptidase	*Geobacillus kaustophilus* HTA426	27	6.2	28	6.5	32	36	gi|56418772	NCBI	MS/MS
8	adenylate kinase cytosolic	*Saccharomyces cerevisiae*	24	6.0	24	6.5	22	26	P07170	SwissProt	MS/MS
9	endonuclease IV	*Escherichia coli*	32	5.4	36	5.0	34	43	P12638	SwissProt	MS/MS
10	peroxisomal NADP-dependent isocitrate dehydrogenase	*Emericella nidulans* (*Aspergillus nidulans*)	47	6.4	48	6.9	16	32	gi|15027827	NCBI	MS
11	glyceraldehyde 3-phosphate dehydrogenase	*Schizophyllum commune*	36	7.0	42	7.5	10	9	P32638	SwissProt	MS/MS
12	alkaline proteinase	*Aspergillus fumigatus*	55	6.0	42	6.5	11	10	P28296	NCBI	MS/MS
13	enolase 1	*Alternaria alternate*	48	5.2			7	220	Q9HDT3		
		Davidiella tassiana	47	5.3	54	5.5	7	220	P42040	SwissProt	MS/MS
		Aspergillus oryzae	47	5.5			4	93	Q12560		
14	nitrogenase iron protein	*Azorhizobium caulinodans*	37	3.0	32	5.0	18	28	P26251	SwissProt	MS
15	enolase 2	*Tuber borchii*	55	4.5	48	5.4	7	52	gi|37147852	NCBI	MS/MS
16	glucosamine:fructose-6-phosphate aminotransferase	*Pseudomonas aeruginosa* PAO1	48	4.4	66	5.7	11	30	gi|9951889	NCBI	MS/MS
17	pyruvate kinase	*Yarrowia lipolytica*	58	6.5	55	6.8	21	28	gi|101735	NCBI	MS

[a] Estimated molecular mass (M_r) and p*I* values estimated from 2-DE PAGE. The p*I* values correspond to the middle of the spots. [b] Theoretical M_r of the matched protein and theoretical p*I* of the matched protein in the database. [c] SwissProt or NCBI accession number. [d] Proteins identified by using MALDI-TOF/TOF MS through PMF or MALDI-TOF/TOF MS and MS/MS.

DISCUSSION

The influence of phosphate limitation for a good pigment-inducing complex medium on the biochemical metabolisms concerning pigment production and culture growth in *Monascus* was investigated using 2-DE and MALDI-TOF/TOF MS. In this instance, *M. pilosus* BCRC 31527 submerged and grown under rice nitrate (R, the control) and NM (R lacking KH_2PO_4) complex media were used. Phosphate limitation to this control medium obviously induced an altered expression of biomass yields and pigment production in the fungus during cultivation (**Figure 2**). The major difference in the use of growth media between our analysis and other nutrient-limited analyses of fungi was that we used complex media rather than defined media. This was due to the fact that a rice source complex medium is considered as a better growth substrate in induction of high pigment productivity in *Monascus* (*11*, *12*, *27*).

The NM medium (7.1 mg phosphate per 100 mL medium) could be considered as a low-phosphate complex medium compared to the control (174.5 mg phosphate per 100 mL medium). We found that the culture of NM medium revealed a higher residual carbon source content in medium than the control after fermentation (**Figure 2A**); consequently, the culture was in a state of lower rate of carbon source consumption compared to the culture of control. Therefore, its carbon source amount to supply its metabolic processes in cells was possibly lesser than the control. Simultaneously, an up-regulation in ALDH and several glycolytic enzymes, including GAPDH, enolase 1, and PK, was expressed in the NM medium fermentation.

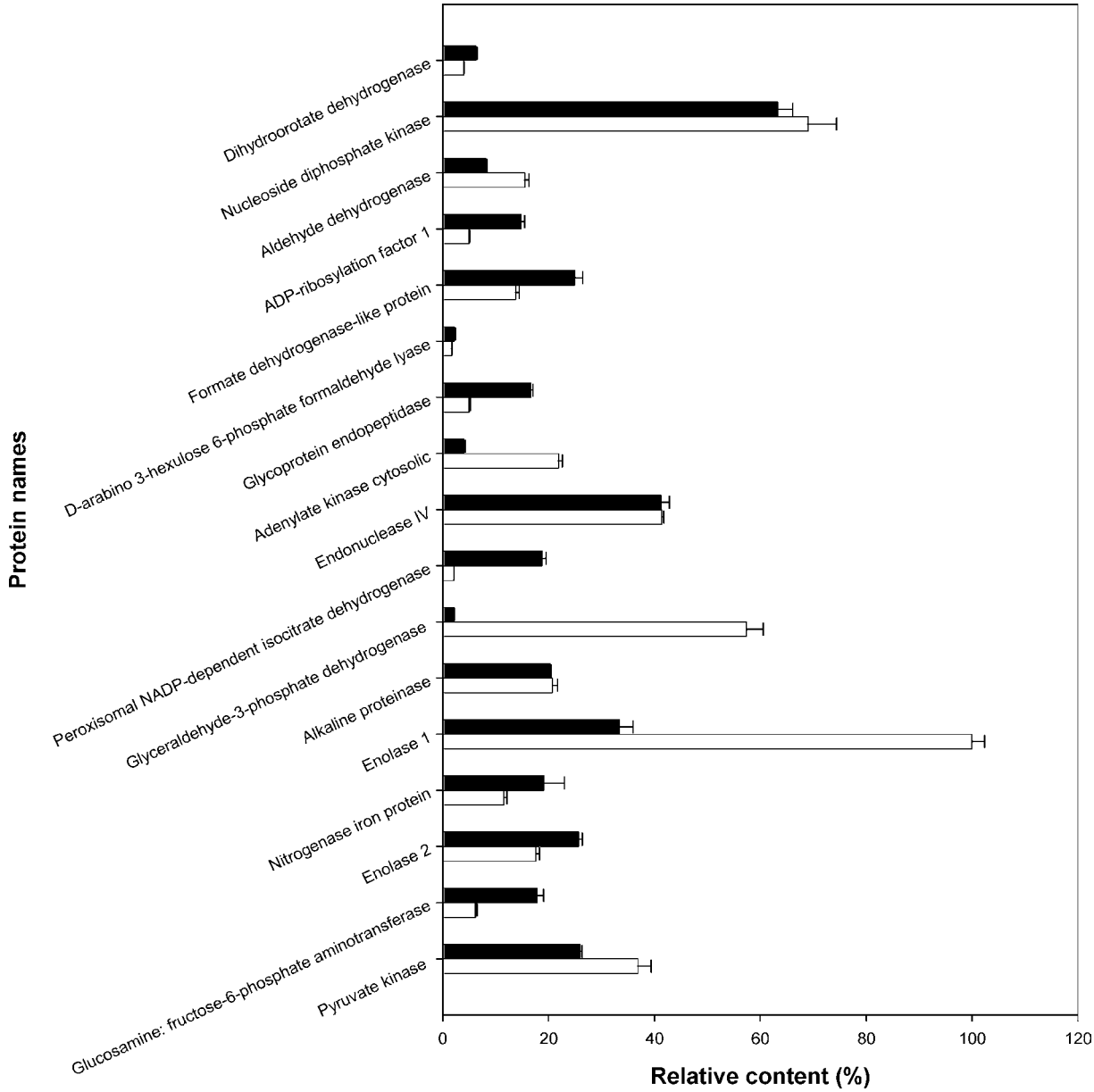

Figure 4. Content of identified proteins comparing the R medium (black bars) to the NM medium (white bars) in cultivation of *Monascus pilosus* BCRC 31527 for 5 days at 32 °C in submerged fermentation. Relative values of protein content were calculated with division of each value by the highest protein content of enolase 1. The results of 2-DE analysis of each growth culture were performed in triplicate, and the data shown were the mean values ± SEM.

Boucherie et al. reported that the synthesis of ALDH, GAPDH, and enolase in *S. cerevisiae* continues throughout the stationary phase in response to glucose limitation (*28*). Moreover, Delgado et al. (*29*) found the cell wall associated GAPDH activity in *S. cerevisiae* increased in response to stress conditions, such as starvation and temperature upshift; similar effects were also observed in *Candica albicans* (*30*). From a comparison of our results with those reports, in our work the increased induction of ALDH and these glycolytic enzymes in *Monascus* was mediated under a carbon source consumption-limited condition in this phosphate limitation medium. In previous studies, many proteins known to be part of the adaptive response are induced by phosphate depletion (*31*, *32*). Phosphate limitation is known to regulate upward of hundreds of different gene expressions in *Escherichia coli* (*33*, *34*), and at the same condition the response in *S. cerevisiae* is mediated by the components of the phosphate signal transduction pathway (PHO pathway) encoded by about 30 genes (*35*−*37*). It was possible that the increased induction of these enzymes might reflect the limitation in carbon utilization, which was induced by phosphate limitation. The occurrence of this induction was presumed to result from the release of carbon catabolite repression due to carbon source exhaustion. Such increased induction of the glycolytic enzymes might allow more efficient utilization of carbon sources by the glycolytic pathway, then going to the subsequent downstream metabolic pathways, such as the citric acid cycle, in response to lesser carbon source supply in the NM medium.

Here, we observed that enolase 1 was expressed conversely with enolase 2 between these two media. Similar to our findings, in *S. cerevisiae*, enolase is encoded by two genes, enolase 1 and enolase 2, that are differentially regulated depending on the carbon source and growth phase (*38*) but are coordinately regulated with other glycolytic enzymes (*39*). In *S. cerevisiae*, one enolase is expressed constitutively while another enolase is induced up to several-fold after the alteration in supplement of glucose to cells (*40*). Changing concentrations of glycolytic

metabolites as the triggering signals for the induction of some of the genes coding for glycolytic enzymes have already been reported in *S. cerevisiae* (*41*, *42*). Therefore, it was possible that the induction of different expression in these two enolases was due to the fact of phosphate limitation-inducing inhibition in carbon consumption in this NM medium. In addition, the PK and AK both were up-regulated in this NM medium. AKs are abundant enzymes that provide the ADP required for oxidative and substrate chain phosphorylations and play an important role in the maintenance of the "energy charge" equilibrium (*43*−*45*). Our study and the study by Huang and O'Shea found AK to have increased induction during phosphate limitation fermentation in fungi (*46*). Since phosphate is essential for many cellular metabolite syntheses, it is possible that phosphate limitation-inducible cellular metabolite regulation is connected to the energy metabolism pathway to coordinate energy transfer reaction balance under this nutrient-limited condition. Consequently, the reason for the up-regulation of these two enzymes together could be explained according to the result noted by Coevoet and Hervagault (*47*), that AK is involved in providing the ADP required for the substrate chain, is in a coupling cycle reaction with PK, and serves to maintain the energy charge equilibrium in the reaction.

In comparison, we observed that the control induced an up-regulation in several other metabolic proteins, including GFAT, ADP-ribosylation factor 1, glycoprotein endopeptidase, and nitrogenase iron protein in cultivated *M. pilosus* BCRC 31527. Our work showed the induction of certain *Monascus* proteins not described by previous proteomic and gene array studies. We were the first to report the protein expression of these four enzymes in *Monascus*. GFAT catalyzes the formation of glucosamine 6-phosphate and is the first rate-limiting enzyme and key regulator of the hexosamine biosynthetic pathway (*48*, *49*). The final product of the hexosamine pathway, UDP-*N*-acetylglucosamine, is an active precursor of numerous macromolecules containing amino sugars and chitin in fungus (*50*). Previous studies reported that increased chitin synthesis for mycelial growth requires activation of the hexosamine biosynthetic pathway by increased expression of this rate-limiting step in the pathway (*51*, *52*). The induction of GFAT may be related to the fact that the cells in the control were in a more rapid growth of cell mass than the NM medium (**Figure 2B**). In addition, ADP-ribosylation factors in *S. cerevisiae* have been implicated as regulators of a large number of essential cellular functions including vesicular traffic, respiration, sporulation, and entry into cell cycle (*24*−*26*). Here, the induction of up-regulation of ADP-ribosylation factor 1 in the control possibly reflected the fermented conditions of the control cells, shown with greater expression in cell biomass and growth regulation. Also, there was a need to maintain greater respiration and sporulation rates relative to the cells of the NM medium in the initial steps of station phase.

Taken together, in this work the differences in induction of certain protein expressions during submerged fermentation between the control and the phosphate-limited NM medium might be *M. pilosus* expressing various responses that allow it to adapt to the different nutrient conditions. However, regardless of the fact that only 23% of proteins in the all cut and analyzed 76 protein spots were identified, this work indicated that the employment of proteomics was a useful technique in understanding the metabolism of *Monascus* in response to different growth media.

Our proteomic comparison of *M. pilosus* grown under these two rice nitrate complex media revealed that the lack of KH_2PO_4 substrate in the complex medium induced an altered expression pattern of this fungus's intracellular proteins. The inductions of altered expression of the identified proteins were involved in glycolysis, energy balance, and other essential metabolisms. Presumably, the diverse expressions of cellular proteins in these two complex media reflected the different cell mass growth patterns in the fungus. The induction of up-regulation of the glycolytic enzymes during fermentation with the lack of KH_2PO_4 substrate in the complex medium suggests the possible involvement of the release of carbon catabolite repression. They may have occurred in response to a shortened carbon source supply in the phosphate-limited complex medium. The deregulation of certain protein expressions during fermentation, in this work, may provide a clue to the identification of the metabolic pathways and fermentation processes in this industrial food fungus.

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