A Possible Role of Peptides in the Growth Enhancement of an Industrial Strain of *Saccharomyces* sp.

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ABSTRACT

Individual addition of a commercially available nutritional supplement and a methanol extract from an industrial *Saccharomyces* sp. strain SMC resulted in the enhanced growth of *Saccharomyces* sp. strain SMC in minimal medium. Isolation of the growth enhancing components from aqueous extracts of the supplement and the cellular extract was performed using reversed-phase, gel filtration, and ion exchange chromatography. Reversed-phase chromatography using Sep-Pak® vac C18 yielded aqueous washes which elicited increased yeast growth. Gel filtration chromatography of the aqueous washes in a group separation mode using Sephadex G25 gave three distinct groups for the nutritional supplement, and four distinct groups for the cellular extract. Fraction groups that exhibited growth enhancing activity also exhibited high absorbances at all three wavelengths of 214, 260, and 280 nm. Two major fractions which tested positive for growth enhancing activity in succeeding experiments were obtained after passing each of the active GFC groups through a Toyopearl SP 550C cation exchanger column. The active component from the cellular extract did not bind to the cation exchanger. The absorbance data at 214 nm (peptide bond experimental absorbance maximum wavelength), the Bradford assay (showing the presence of proteinaceous matter), and the active component's inclusion in the Sephadex G25 fractionation range of 1-5 kDa (characteristic of small peptides) suggest that the growth enhancing components of the nutritional supplement and methanol cell extracts are peptides.

Keywords: brewer's yeast, peptides, organic extraction, chromatography, Bradford assay, nutritional supplement

INTRODUCTION

In the alcoholic beverage industry, the biochemical cycle by which yeast metabolizes simple sugars into ethanol and other flavor compounds has always been closely watched for opportunities in which the brewer may intervene, either by manipulation of biological components or changes in process parameters. Differences between what is known as the traditional brewing process as compared to modern methods are fast increasing in number. The progression of the

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industry as a whole from small cottage breweries to the large factories has been brought about by increasing competition in a lucrative market wherein consumers demand the highest quality product at the most affordable price (Iserentant, 1995).

Yet, even with the demand for change, brewers are cautious as to how they may alter their time-honored recipes, given consumer response to product changes. Of current concern not only in alcoholic beverage but also in all other food industries are the advantages and disadvantages of using molecular techniques to improve current procedures and products. To the uninformed consumer, molecular biology almost always translates to genetic manipulation, a controversial issue that is currently negative in public perception. The industry is, thus, reluctant to employ molecular techniques given the above premise. However, deoxyribonucleic acid (DNA) is not the only biomolecule that may be manipulated for process or product improvement; there still are the downstream components of molecular biology's central dogma– RNAs, peptides, and proteins.

Improvement of the production medium is one way to increase the product. We have been interested on the possible role of peptides as enhancers to the growth and fermentation performance of a *Saccharomyces* sp. industrial strain used primarily for beer production. Of several nitrogenous sources peptides are the most promising of all alterable factors in sugar substrate biochemistry (da Cruz et al., 2002; Patterson & Ingledew, 1999). The next step would be to test its activity on the actual industrial sugar substrates used by brewers, prior to possible inclusion in the standard brewer's recipe.

This study deals with a potential biological parameter that may be exploited for industrial application. The study was conducted with the following specific objectives:

(i) To test the commercially-available supplement and the yeast cell extract for growth enhancing activity.

(ii) To examine the chemical nature of the supplement and yeast cell extract.

(iii) To test the isolated fractions for growth enhancing activity.

MATERIALS AND METHODS

Saccharomyces growth and maintenance

An industrial *Saccharomyces* sp. brewer's yeast strain was provided by San Miguel Corporation's Beer Division (SMCBD, Mandaluyong City, Metro Manila, Philippines) and referred here as *Saccharomyces* sp. strain SMC. Upon receipt of the yeast it was subcultured onto a YEPD-agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar agar) plate. The plate culture was then stored at 4°C for a maximum of one month, after which subculturing onto a new plate was done. Inoculum for the liquid culture was obtained from the YEPD agar plate culture. Each colony was suspended in 1 mL sterile distilled deionized water (sddH_2O) before inoculation into the media.

Preparation of crude nutritional supplement and yeast methanol extract

A liquid sample of yeast "peptide supplement" (as advertised) was provided by SMCBD in a sealed dark brown bottle at room temperature. The liquid supplement was diluted with sddH_2O in a 1 part supplement: 2 parts sddH_2O ratio. The sample was then centrifuged for 15 minutes at 10,000 x g and 4°C. Supernatant was then filtered using a 0.22 µm membrane filter. The clarified supernatant was stored in sealed, sterile conical tubes at 4ºC until further use.

Freshly-prepared industrial brewer's yeast (*Saccharomyces* sp. strain SMC) slurry obtained from SMCBD was washed with distilled deionized water (ddH_2O) until the supernatant was colorless. Whole yeast cells were pelleted and collected from the slurry after centrifugation at 10,000 x g for 15 minutes at 4ºC. The yeast cells were allowed to freeze at -70ºC and were lyophilized until fully dry. Organic extraction was then performed by stirring 7:3 methanol:water solution (70% methanol) into the dried yeast cells until a homogeneous suspension was obtained. The yeast

suspension was centrifuged at 10,000 x g for 15 minutes at 4ºC and the supernatant was collected and stored as the crude extract. The extraction process was performed twice on the same batch of samples.

The crude yeast extract was dried by centrifugation under vacuum with heating at 45ºC. The samples were resuspended in sddH_2O, sterilized using a 0.2 µm filter and stored at 4ºC.

Sample purification and analysis

The nutritional supplement and the crude yeast cell extract were individually purified and analyzed as follows:

Reversed-phase chromatography: Sep-Pak® C18

Sep-Pak® vac 6 cc cartridges (Waters Corporation, Massachusetts, USA) containing a C18 matrix were used in a solid phase extraction (SPE) mode relying on reversed-phase chromatography (RPC) principles. An aqueous wash fraction (using ddH_2O as eluent) and an organic wash fraction (using 70% v/v methanol as eluent) were separately obtained from the samples. The two fractions were then lyophilized and individually resuspended in ddH_2O and later assayed for growth enhancing activity.

Gel filtration chromatography: Sephadex G25

Gel filtration chromatography (GFC) in a batch separation mode was performed using a Sephadex G25 solid matrix, having a fractionation range of 1-5 kDa. A glass column with 50 cm length x 1.5 cm internal diameter was packed with Sephadex G25 superfine-grade resin in a 4ºC cold cabinet. Column efficiency and void volume were checked and measured using Blue Dextran 2000 (Amersham Biosciences, Sweden). Three mL of the active fraction from the RPC run was then loaded onto the GFC column and eluted with ddH_2O. The run proceeded at 0.5 mL/minute. Four mL-sized fractions were collected and monitored for absorbances at 214, 260, and 280 nm. Upon analysis of absorbance data from the GFC run, fraction groups were selected and pooled according to distinctions in absorbance peaks. The resultant groups were then lyophilized and resuspended in ddH_2O, before being assayed for growth enhancing activity.

Ion exchange chromatography: Toyopearl SP 550C

Ion exchange chromatography (IEX) using a Toyopearl SP 550C cation exchanger resin was performed using a Bio-Rad Econo System fast protein liquid chromatography (FPLC) setup (Bio-Rad Laboratories, Inc., USA). A glass column with 9 cm length x 1.5 cm internal diameter was packed with resin at room temperature.

Five hundred µL of the active GFC group was loaded onto the IEX column and eluted initially with ddH_2O (0% salt). A linear gradient was then formed by the FPLC, from 0% to 100% of 0.5 M NaCl salt solution. Gradient start and end points were recorded and are noted in the IEX chromatogram. The run proceeded at 1.5 mL/minute, and fractions were collected at 3 mL/fraction. The fractions were monitored for absorbances at 214, 260, and 280 nm.

Protein assay

The Bradford assay (Bradford, 1976) was utilized in order to verify the presence of proteins and/or peptides. Aliquots of the test samples in volumes from 5 to 50 µL were each brought to 100 µL sample volume using 0.15 M NaCl solution. One mL of Bradford reagent (Sigma) was added to each sample in microcentrifuge tubes. The tubes were then vortexed and incubated at room temperature for at least two minutes, after which sample color change was evaluated and compared to a negative control.

Influence on growth of Saccharomyces sp. SMC strain

The influence of nutritional supplement and yeast methanol extract on the growth of *Saccharomyces* sp. strain SMC was examined using a liquid minimal medium (0.67% w/v yeast nitrogen base without amino acids and ammonium sulfate, 2% w/v glucose). Five hundred µL of the test sample was added into cotton-plugged 250 mL Erlenmeyer flasks, each containing

100 mL sterile liquid minimal medium. Inoculation with a single colony of the yeast followed. The increase in cell mass was measured over time by monitoring the absorbance at 660 nm (Dillemans et al., 1999) of 1 mL aliquots of the culture. The purity of the culture after the addition of the supplement was determined by spread plating and inspection of colony morphology.

RESULTS AND DISCUSSION

Growth of *Saccharomyces* sp. industrial strain SMC with or without the supplements

The addition of the nutritional supplement resulted in considerable growth enhancement of the industrial strain *Saccharomyces* sp. (SMC) as shown by its growth curve compared to that of the negative control (Fig. 1). Similarly, the cellular extract enhanced the growth of the same yeast strain in minimal medium compared to the control (Fig. 2). The cell growth enhancement is most likely caused by nitrogenous additives, because the culture medium is nitrogen-deficient. The water used was distilled and deionized, lessening the likelihood that ammonium salts could have been introduced via the water component.

Previous work by Patterson and Ingledew (1999) showed that mixtures of nitrogen sources, amino acids, ammonia, allantoin, and urea were preferred by yeast

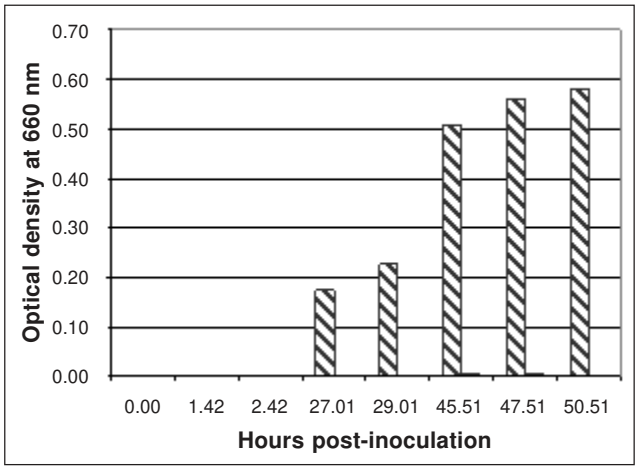

Fig. 1. Effect of nutritional supplement on the growth of *Saccharomyces* sp. SMC strain with (\\) or without (■) the prepared minimal supplement. *x* axis = time (hours); *y* axis = absorbance (660 nm).

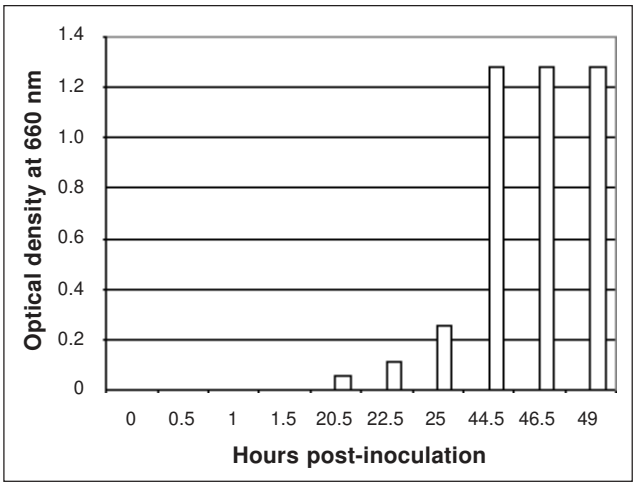

Fig. 2. Effect of cell extract on the growth of *Saccharomyces* sp. SMC strain. Liquid minimal medium was with ( ) or without (■) crude yeast extract. *x* axis = time (hours); *y* axis = absorbance (660 nm).

and appeared to inhibit the utilization of dipeptide from culture medium. Single amino acids were better sources of nitrogen than were single sources of each homodipeptide. In the same study, it was observed that complex nitrogen mixtures (3 amino acids and 3 dipeptides), the yeast *Saccharomyces cerevisiae* NCYC 1324 simultaneously used both amino acids and peptides as sources of nitrogen. These previous reports suggest that in the absence of other preferred nitrogen sources, dipeptides enhance yeast growth. In this study, addition of putative peptides present in the nutritional supplement and cell extracts, probably served as a crucial source of nitrogen because the yeast cells were grown in minimal medium. The presence of the putative peptides as N source resulted in significant growth enhancement compared to the control.

Sample analysis

The methods used in this study are standard protocols for the isolation, purification, and characterization of proteins or peptides. Whereas larger proteins are commonly rendered inactive by the methanol wash because they are denatured by the same hydrophobic properties of methanol, methanol extraction is generally the preferred way to extract peptides, because their small sizes keep their structure from being disrupted by the hydrophobic interaction of methanol. The purification strategies were devised taking into

consideration the novel nature of the peptide(s) in question and the relevance of each procedure to the biomolecule's presumed structural complexity.

Protein assay of the samples

Aliquots from the prepared nutritional supplement all qualitatively registered a brick red to blue colorimetric reaction upon addition of the Bradford reagent, suggesting the presence of proteinaceous matter. The same brick red to blue color change was manifested using the yeast cell extract fractions in the protein assay, thus, also indicating the presence of proteinaceous matter. The data using the Bradford assay, however, could not accurately quantify the protein contents of the samples because different amino acid compositions have different absorbance readings depending on the binding of the Coomassie® dye. Selection of an appropriate standard protein is necessary to obtain an approximate amount of proteins in an unknown sample. This study began with a totally unknown active component, and a correct standard for quantitation could not be selected at this point (Tal et al., 1985).

Sample purification and fraction analysis

Liquid chromatography techniques employed as part of the purification strategy include reversed-phase chromatography, gel filtration chromatography, and ion exchange chromatography. Component elution was monitored by taking the absorbance values (at 214, 260, and 280 nm) of the fractions from the chromatography steps (except the RPC step since it was in a batch separation mode). The three wavelengths were used based on theoretical absorbance profiles for proteins in general, certain amino acids with aromatic side chains, and the peptide backbone (280, 260, and 214 nm, respectively; Mathews & van Holde, 1996).

Reversed-phase chromatography (RPC) and gel filtration chromatography (GFC) fractions were assayed for growth enhancement activity and tested for the presence of proteinaceous matter using the Bradford assay. Ion exchange chromatography was performed on the active GFC fraction of the nutritional supplement and the active component in the cell extract, revealing two major molecular groups present according to positive or negative charge in the first and that the active component did not bind to the cation, in the latter.

Reversed-phase chromatography: Sep-Pak® C18

Solid Phase Extraction (SPE)-RPC using the Sep-Pak® C18 cartridges gave two fractions from a batch mode: an aqueous (ddH_2O) wash and an organic (70% methanol) wash, for both commercial nutrition supplement and methanol extracted samples. Upon Bradford analysis, the two washes of each sample retained the brick red to brown color of the original clarified supernatant. It was observed, however, that only the aqueous wash for both samples exhibited considerable growth enhancing activity turbidimetrically (Figs. 3 and 4) and by viable plate count (data not shown).

The active components for both samples did not adsorb to the hydrophobic C18 matrix of the Sep-Pak® cartridge, suggesting that they must have weak hydrophobicity, or that they may even be hydrophilic. Based on the Sep-Pak® product literature, an analyte can either adsorb to the matrix with contaminants eluting in the aqueous wash, or an analyte can elute with the aqueous wash with contaminants adsorbing to the matrix. It is likely that the second case holds in this study, because the organic 70% methanol wash appears

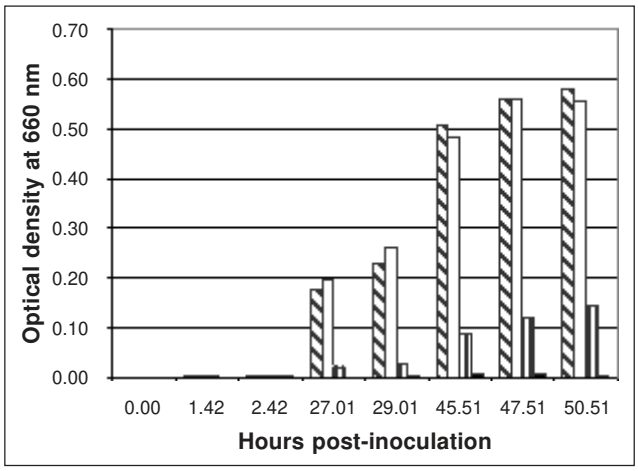

Fig. 3. Effect of nutritional supplement's Sep-Pak® fractions on the growth of *Saccharomyces* sp. SMC strain. The samples tested were the aqueous Sep-Pak® wash ( ), the prepared nutritional supplement (\\), the 70% methanol wash (|||), and the control (■). *x* axis = time (hours); *y* axis = absorbance (660 nm).

not to contain significant active component and exhibited only a low level of growth enhancement.

Gel filtration chromatography: Sephadex G25

The GFC run resulted in three distinct fraction groups for the nutritional supplement (Fig. 5) and four distinct fraction groups for the cell extract (Fig. 6). Fractions from each of the major groups were pooled together on the basis of the continuity of their absorbances at 214, 260, and 280 nm. Only the second GFC groups of the nutritional supplement and of the cellular extract exhibited growth enhancing activity turbidimetrically (Figs. 7 and 8) and by viable cell count (data not shown).

Ion exchange chromatography: Toyopearl SP 550C

IEX was performed to investigate if the ionic interaction-based chromatography step could further purify the second GFC groups of the two samples. As

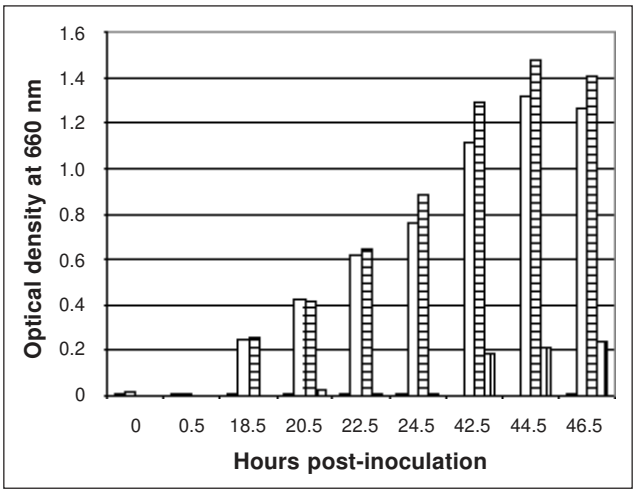

Fig. 4. Effect of cell extract Sep-Pak® fractions on the growth of *Saccharomyces* sp. SMC strain. The samples tested were the aqueous RPC (=), organic RPC fraction (|||), the crude yeast extract ( ), and the control (■). *x* axis = time (hours); *y* axis = absorbance (660 nm).

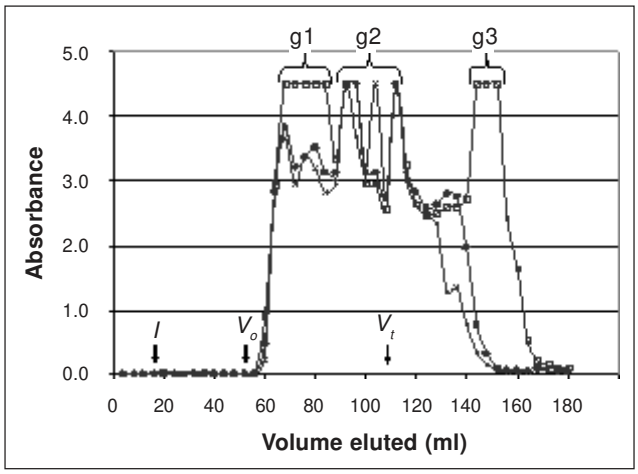

Fig. 5. Sephadex G25 GFC chromatogram (batch separation mode) of the nutritional supplement. Fraction absorbance values were read at 214 (□), 260 (•), and 280 nm (**x**). Fractions were pooled into three groups (g1, g2, g3). *I* = sample injection point, V_o = void volume, V_t = total column volume.

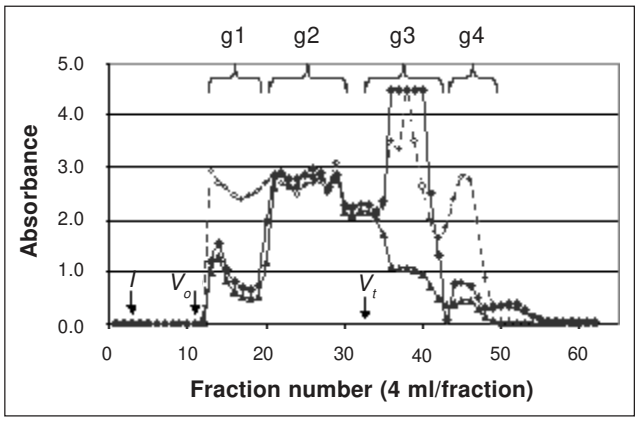

Fig. 6. Sephadex G25 GFC chromatogram (batch separation mode) of the cell extract. The absorbance of the fractions were read at 214 (O), 260 (♦), and 280 nm (▲).

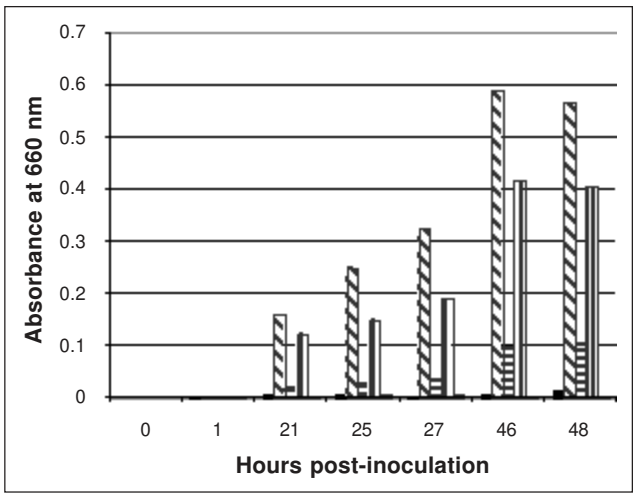

Fig. 7. Effect of nutritional supplement GFC fractions on the growth of *Saccharomyces* sp. SMC strain. The samples tested were the active Sep-Pak® fraction (\), GFC groups g1 (=), g2 (|||), g3 ( ), and negative control (■). *x* axis = time (hours); *y* axis = absorbance (660 nm).

may be seen in the chromatograms, there were only two distinct peaks (Figs. 9 and 10). This suggests that IEX may be used as a purification step to clean the sample. Furthermore, both IEX groups 1 and 2 of the cellular extract showed growth enhancement activity compared to that of the negative control (Fig. 11). Only the enhancement brought about by IEX group 1 is comparable to the crude extract's activity. The enhancement caused by group 2, though not as significant, may have been brought about by other positively charged molecules. These data from the chromatographic separation suggests that the active components of the cellular extract are negatively charged molecules.

Previous work by Patterson and Ingledew (1999) showed that mixtures of nitrogen sources, amino acids, ammonia, allantoin, and urea were preferred by yeast

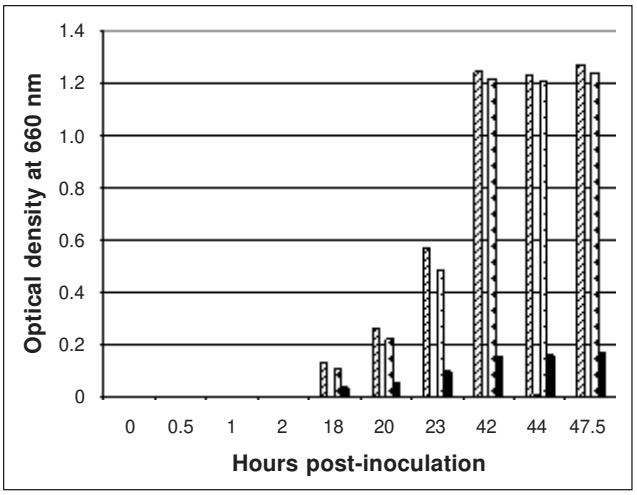

Fig. 8. Effect of cell extracts GFC fractions on the growth of *Saccharomyces* sp. SMC strain. The samples tested were the negative control (=), yeast cultures with aqueous RPC fraction (/), GFC groups g1 (\), g2 (♦), g3 (■), and g4 ( ). *x* axis = time (hours); *y* axis = absorbance (660 nm).

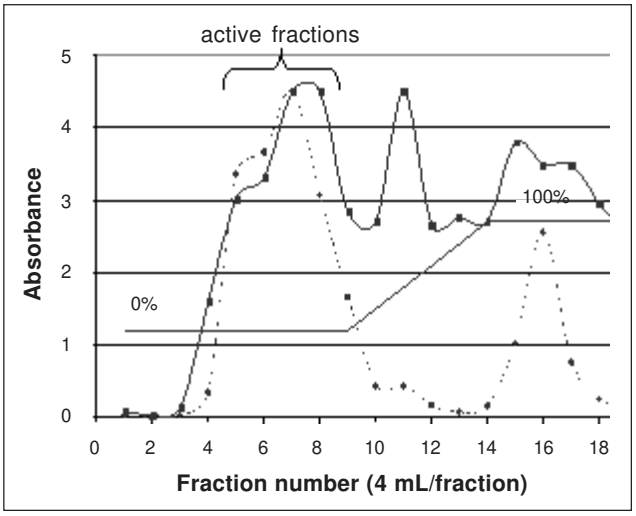

Fig. 10. Ion exchange chromatogram of GFC group g2 (cell extract). The absorbances were read at 214 nm (■) and 280 nm (•). The profile of the salt gradient used for elution is shown as the heavy line (—).

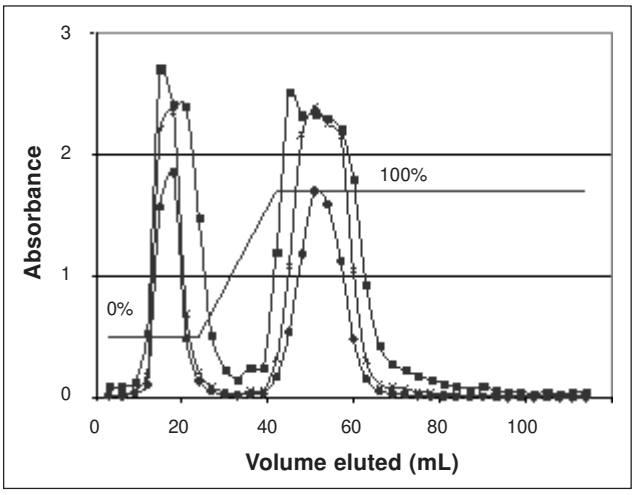

Fig. 9. Ion exchange chromatogram of GFC group g2 (nutritional supplement). The linear gradient's progression from 0% to 100% 0.5 M NaCl is marked by the straight line. The absorbances were read at 214 nm (■), 260 nm (**x**), and 280 nm (=).

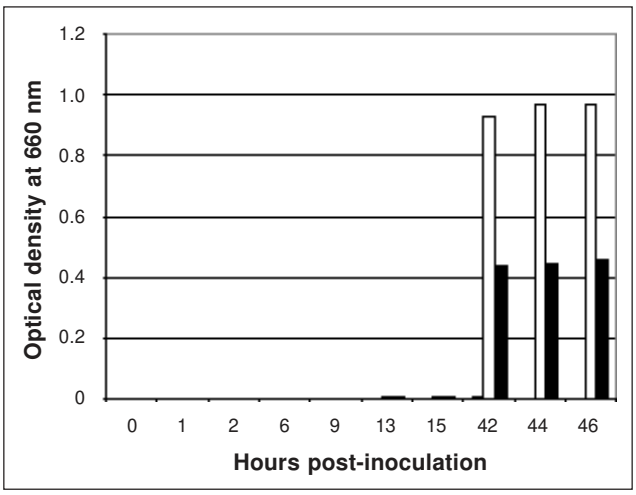

Fig. 11. Effect of cell extract IEX fractions on the growth of *Saccharomyces* sp. SMC strain. The samples tested were the yeast cultures with IEX fraction 1 ( ) and 2 (■) and the control (|||). *x* axis = time (hours); *y* axis = absorbance (660 nm).

and appeared to inhibit the utilization of dipeptide from culture medium. Single amino acids were better sources of nitrogen than were single sources of each homodipeptide. In the same study, it was observed that complex nitrogen mixtures (3 amino acids and 3 dipeptides), the yeast *Saccharomyces cerevisiae* NCYC 1324 simultaneously used both amino acids and peptides as sources of nitrogen. These reports suggest that in the absence of other nitrogen sources, dipeptides enhance yeast growth. In this study, putative peptides probably served as crucial source of nitrogen because the yeast cells were grown in minimal medium, resulting in significant growth enhancement when nutritional supplements or cell extracts were added compared to the control.

CONCLUSION

Enhanced growth of *Saccharomyces* sp. strain SMC in minimal medium was observed upon the addition of either commercially available nutritional supplement or a methanol extract from an industrial *Saccharomyces* sp. strain SMC. Analysis of the nutritional supplement and yeast methanol extract revealed significant absorbance at 214 nm wavelength (peptide bond experimental absorbance maximum wavelength), the presence of proteinaceous matter based on protein assay, and the active component's inclusion in the Sephadex G25 fractionation range of 1-5 kDa (characteristic of small peptides), suggesting that the growth enhancing components of the nutritional supplement and methanol cell extracts are peptides.

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