การแยกเชื้อจุลินทรีย์ที่มีกิจกรรมของเอนไซม์อินเวอร์เทสสูง

Isolation of Microorganisms Exhibiting High Invertase Activity

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บทคัดย่อ

อินเวอร์เทสเป็นเอนไซม์ที่เร่งปฏิกิริยาการเปลี่ยนน้ำตาลซูโครสให้เป็นน้ำตาลกลูโคสและฟรุคโตส ในงานวิจัยนี้ได้ทำการแยกจุลินทรีย์ที่มีกิจกรรมของเอนไซม์อินเวอร์เทสสูงจากตัวอย่างน้ำอ้อย ท่อนอ้อย และดิน จากภาคตะวันออกเฉียงเหนือของประเทศไทย โดยทำการคัดเลือกโคโลนีที่มีการเจริญดีในอาหารแข็ง PCA, MRS และ ME ที่มีน้ำตาลซูโครส 20 เปอร์เซ็นต์ ได้เชื้อจำนวน 148 โคโลนี และได้ทำการคัดเลือกเชื้อที่มีการเจริญดีในอาหารเหลวสูตรอุดม และสูตรปรับต่ำที่มีซูโครส 10 เปอร์เซ็นต์ ที่อุณหภูมิ 30 องศาเซลเซียส สามารถแยกได้เชื้อที่มีการเจริญดีเป็นยีสต์ 7 สายพันธุ์ ได้แก่ *Candida guilliermondii* 3M4.1, *Cryptococcus humicolus* (*Candida humicola*) 5M2 และ 6M3.1, *Saccharomyces cerevisiae* MM2.1, 6M4.1, 7M3.1.1 and 7M3.2.1 ซึ่งมีอัตราการใช้น้ำตาลซูโครสเท่ากับ 13.45, 20.38, 19.96, 12.23, 22.97, 13.98, และ 20.37 กรัมต่อลิตรต่อชั่วโมง ตามลำดับ ได้ทำการตรวจสอบความจำเพาะของเอนไซม์อินเวอร์เทส โดยการใช้น้ำตาลแรฟฟิโนสและเมเลซิโตสเป็นสารตั้งต้น พบว่ายีสต์ทุกสายพันธุ์มีกิจกรรมของเอนไซม์อินเวอร์เทส และพบอยู่ทั้งในเซลล์และนอกเซลล์ ยีสต์ทุกสายพันธุ์มีกิจกรรมของเอนไซม์อินเวอร์เทสที่พบในเซลล์ (30-90 U/mg) สูงกว่าที่พบนอกเซลล์ (0.1-0.9 U/mg) *S. cerivisiae* 6M4.1 มีกิจกรรมของเอนไซม์อินเวอร์เทสสูงสุดทั้งที่พบอยู่ภายในเซลล์และนอกเซลล์ เท่ากับ 90.96 U/mg และ 0.95 U/mg ตามลำดับ ที่ระยะคงตัว และบ่งชี้ว่าเป็นสายพันธุ์ที่มีศักยภาพในการผลิตเอนไซม์อินเวอร์เทส

Abstract

Invertase catalyzes sucrose into glucose and fructose. In this research, microorganisms exhibiting high invertase activity were isolated from sugarcane juice, sugarcane stalks and soil from northeastern Thailand. The 148 colonies on the PCA, MRS and ME agar media containing 20% sucrose were firstly selected. The isolates with high growth potential were further screened in complete and basal salt minimum liquid media containing 10% sucrose at 30 °C. The microorganisms isolated from sugarcane juice were seven isolates of yeasts. The results showed that *Candida guilliermondii* 3M4.1, *Cryptococcus humicolus* (*Candida humicola*) 5M2 and 6M3.1, *Saccharomyces cerevisiae* MM2.1, 6M4.1, 7M3.1.1 and 7M3.2.1 had a sucrose

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consumption rate of 13.45, 20.38, 19.96, 12.23, 22.97, 13.98, and 20.37 g/l/h, respectively. All the isolates exhibited extracellular and intracellular invertase activities during the early log phase and stationary phase. The crude intracellular invertase activities (30-90 U/mg) of all isolates were higher than extracellular invertase activities (0.1-0.9 U/mg). *S. cerevisiae* 6M4.1 had both the highest activities of intracellular (crude cell extract) and extracellular invertase activity of 90.96 U/mg and 0.95 U/mg at the stationary phase respectively. The results indicate that *S. cerevisiae* 6M4.1 has the potential for invertase production.

คำสำคัญ: อินเวอร์เทส ยีสต์ น้ำอ้อย
Keywords: invertase, yeast, sugarcane juice

Introduction

There is a great deal of sugarcane cultivation in Thailand, especially in the northeastern part. The income from sugar export was approximately 28,000 million baht ($ 700 million) in 2005 (Office of the Cane and Sugar Board, 2005). However, the price of refined sugar in Thailand is as low as $ 0.5 per kilogram and so a method to increase the value of the product is needed. An alternative way to get higher value is to change sucrose into monosaccharides such as glucose, fructose, sorbitol or mannitol, lactic acid, and invertase for many food industries such as inverted sugar and artificial honey etc.

Sucrose is hydrolyzed into glucose and fructose generally by saccharase or sucrase. Saccharase specificities are invertase (EC.3.2.1.26, β-D-fructofuranosidase) and α-D-glucosidase (EC.3.2.1.20) which hydrolyze sucrose at the fructose and glucose sides respectively to free reducing sugars. Invertase is widely found in bacteria, fungi, higher plants, and some animals (Ishimoto and Nakamura, 1997; Lee and Sturm, 1996; Vorster and Botha, 1998). Some kinds of microorganisms can produce extracellular and intracellular invertases. Invertase or beta-fructofuranosidase is commonly found in *Saccharomyces cerevisiae* (Matula et al., 1980). *Streptococcus mutan* SL-1 and *Candida utilis* produce both intracellular and extracellular invertases at late log phase and stationary phase (Osborne et al., 1975; Belcarz et al., 2002) whereas *Clostridium pasteurianum* and *Aspergillus ochraceus* can produce only intracellular or extracellular invertase, respectively (Laishley, 1975; Ghosh et al, 2001). Most invertases are glycoprotein and have different molecular masses and forms depending on the kind of microorganisms. Bacterial invertase is mostly found as a monomer subunit of glycoprotein such as 58 kDa in *Lactobacillus reuteri* CRL1000 (DeGines et al., 2000) and 48 kDa in *Streptococcus mutans* CS (Maynard and Kuramitsu, 1979). The molecular mass of fungal invertases is large, for example 82-251 kDa in *Aspergillus niger* A 50023 (Hocine et al., 1999); 270 kDa in *Saccharomyces cerevisiae* (Matula et al., 1980); and 60 and 300 kDa in *Candida utilis* (Belcarz et al., 2002). A microorganism which has a monomer subunit of extracellular invertase is ideal desirable for separation and purification.

The aims of this research were, therefore, to isolate the microorganisms exhibiting high invertase activity from sugarcane juice, trashed stalk and soil in the northeastern part of Thailand. The extracellular and intracellular invertases from each isolate were also studied.

Material and Methods

1. Microbial samples and chemicals

There were 7 samples of sugarcane juice from Mitr Phuveing Sugar Factory, 11 samples of sugarcane stalks and 6 samples of soil from the northeastern part of Thailand. All chemicals used were of analytical grade.

2. Isolation and identification of microorganisms

Microorganisms exhibiting high invertase activity were primarily screened by culturing microbial samples in three kinds of solid media containing 20% sucrose : plate count agar (PCA) for general microorganisms (Nickelson and Finne, 1992), deMan Rogosa and Sharpe (MRS) for lactic acid bacteria (deMan et al.,1960) and malt extract (ME) for yeasts and molds (Albert et al.,1980). The mucoid colonies were selected and subsequently screened by growing in modified complete medium (CM) (Stein, 1973) and modified basal salt (BS) minimum liquid medium (Yeager, 1999) containing 10% sucrose and 0.0018% phenol red as an indicator. The microrganisms changing the color of phenol red within 4 hours were subsequencely selected by growing in the CM liquid medium containing 10% sucrose aerobically by shaking at 160 rpm, 30 °C. Rapidly growing microorganisms were selected and identified by physiological and biochemical characteristics at the Thailand Institute of Scientific and Technological Research (TISTR).

3. Growth and time course of sucrose consumption

The selected isolates were aerobically grown in CM containing 10% sucrose by shaking at 160 rpm, 30 °C. The growth of these isolates was observed by spectrophotometry (OD 600 nm). The sucrose concentration from cultures was determined by enzymatic assay using commercial purified invertase (Fluka). Invertase assay was performed by determining the liberated reducing sugars by 3, 5-dinitrosalicylic acid (DNS) (Chaplin, 1987). The total volume of 4.5-ml assay mixture contains 0.5 ml of the purified invertase (7.5 U in 0.1 mM acetate buffer, pH 4.6); 2.5 ml of 0.1 M sucrose; and 1.5 ml of 10 mM acetate buffer pH 4.6. The mixtures were incubated at 37 °C for 30 min. The reactions were stopped by boiling the mixture with 2 ml of DNS solution for 10 min. The liberated reducing sugars were determined at A 540 nm and calibrated with sucrose standard (0-0.2 mM). The sucrose consumption rates of yeasts were calculated from sucrose utilization in one liter of culture at log phase per hour (g/l/h).

4. Characterization of extracellular and intracellular invertases

The isolates were grown in modified CM containing 10% sucrose at 160 rpm, 30 °C. One ml of culture suspension each at the early log, mid-log and stationary phases were collected and centrifuged at 8,000 g, 4 °C for 10 min. The supernatant was assayed for extracellular invertase. The intracellular invertase activity was determinined from intact cells. The harvested cells were resuspended and washed once in 10 mM acetate buffer pH 4.6. The invertase activity was measured using the method mentioned in 3. The reducing sugars were determined at A 540 nm and calibrated with glucose standards

(0-0.4 mM). One unit of invertase activity was defined as the amount of enzyme that hydrolyzes sucrose to 1μmole of glucose per mg protein per minute at 37 °C and pH 4.6. Protein concentration was determined by the Lowry method (Lowry et al., 1951). Statistical analysis of invertase activities were performed by ANOVA.

5. Invertase specificity assay

The invertase specificity can be identified by using trisaccharide raffinose and melezitose as substrates. Sucrose is hydrolyzed by invertase (EC.3.2.1.26, β-D-fructofuranosidase) on the fructose side and α-D-glucosidase (EC.3.2.1.20) on the glucose side to fructose and glucose. Invertase can catalyze the detachment of the terminal fructose side of raffinose (galactose-glucose-fructose) to free fructose, while glucosidase can catalyze the detachment of the terminal glucose side of melezitose (glucose-fructose-glucose) to free glucose (Gabriel and Wang, 1969; Chris, 1974).

The intracellular invertase from crude cell extract of each isolate grown at the stationary phase was subjected to invertase specificity determination using trisaccharides (raffinose and melezitose) and sucrose as substrates. The yeast cell culture (100 ml) was harvested by centrifugation and ground with the aluminum oxide in 20 ml of proteinase inhibitor solution (62.5 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM PMSF). The crude cell extract was separated by centrifugation at 8,000 g, 4 °C for 10 min. The proteinase inhibitor solution was removed and the crude cell extracts were concentrated by centricon (molecular weight cut off at 10 kDa). The remaining crude cell extract from the upper fraction in centricon was adjusted to 10 ml by 10 mM acetate buffer, pH 4.6. The samples of crude enzyme solution were subjected to invertase activity assay with 0.1 M raffinose, melezitose, and sucrose as substrates. One unit (U) of invertase activity was defined as the amount of enzyme that hydrolyzes substrate to 1 μmole of glucose as a reducing sugar per mg protein per minute at 37 °C and pH 4.6.

Results and Discussion

1. Isolation and identification of microorganisms

The 148 isolates showing mucoid colonies were firstly selected from PCA, MRS and ME agar media containing 20% sucrose. The 24 isolates were secondarily screened by growing in complete liquid medium containing 10% sucrose. The best 7 isolates with high growth potential were selected by aerobically growing in complete medium and basal salt minimum liquid medium containing 10% sucrose. All the isolates were yeasts from sugarcane juice including *Candida guilliermondii* 3M4.1, *Cryptococcus humicolus* (*Candida humicola*) 5M2 and 6M3.1, and *Saccharomyces cerevisiae* MM2.1, 6M4.1, 7M3.1.1 and 7M3.2.1.

2. Growth and time course of sucrose consumption

The growth phases of 7 isolates in CM medium containing 10% sucrose were similar, showing early, mid log, and stationary phases at approximately 10, 20 and 30 hours (h), respectively (Figure 1). The biomass at stationary phase of these yeasts was very high (OD 600 nm = 15-22 units). The time course of reducing sugar liberation and a decrease in sucrose concentration were correlated with the growth of yeasts as shown in Figure 2 for *S. cerevisiae* 6M4.1 as a representative. Sucrose concentration decreased very rapidly during the early log to mid log phases and became undetectable at the stationary phase. Reducing sugar was found to

correlate with the growth and started decreasing after the mid log phase. *S. cerevisiae* 6M4.1, *C. humicolus* 5M2, *S. cerevisiae* 7M3.2.1, *C. humicolus* 6M3.1, *S. cerevisiae* 7M3.1.1, *C. guilliermondii* 3M4.1, and *S. cerevisiae* MM2.1 had a sucrose consumption rate of 22.95, 20.41, 20.37, 19.99, 13.98, 13.46 and 9.28 g/l/h, respectively. Yeast could utilize sucrose more rapidly than bacteria. *Leuconostoc mesenteroides* strain 3A, a lactic acid bacterium commonly found as a contaminant in many sugar factories, could rapidly utilize sucrose at 8.46 g/l/h at 30 °C within the first 6 hours of growth (DeGuglielmone et al., 2000). Therefore, yeast causes the problem of contamination which is harmful to the sugar industry.

3. Characterization of extracellular and intracellular invertase activities

All of the 7 yeast isolates showed both extracellular and intracellular invertase activities. The enzyme activities from intact cells of each isolate were found at all the three phases of growth (Figure 3 and Figure 4). It was found that *C. humicolus* 5M2 and *S. cerevisiae* 7M3.2.1 exhibited the highest extracellular invertase activity at the mid log phase (20 h) whereas *S. cerevisiae* 6M4.1 showed the highest enzyme acitivity at the stationary phase (30 h). The intracellular invertase activity from the intact cells of *C. guilliermondii* 3M4.1, *C. humicolus* 5M2 and 6M3.1, and *S. cerevisiae* 7M3.2.1 remained high between the mid log phase and the stationary phase. However, those of *S. cerevisiae* MM2.1, 6M4.1, and 7M3.1.1 were high only at the early log phase. At the stationary phase (30 h), the activities of intracellular invertase (30-90 U/mg) from the crude cell extracts (Figure 5A) of all isolates were higher than those of extracellular invertase (0.17-0.95 U/mg) (Figure 5B). The acitivities of intracellular invertase from the intact cell as shown in Figure 5C (0.4-3.5 U/mg) were lower than those of intracellular invertase from the crude cell extracts as shown in Figure 5A (30-90 U/mg). However, the enzyme activities of each isolate at 30 h from the crude cell extract (Figure 5A) and the intact cell (Figure 5C) were highly correlated. The results show that *S. cerevisiae* 6M4.1 had the highest extracellular activity (0.95 U/mg) and intracellular (90.96 U/mg) invertase activities at the stationary phase (30 h).

For invertase specificity, all the crude enzyme of the 7 yeast isolates could hydrolyze only raffinose (Table 1). This indicates that all of the isolates exhibit only invertase activity.

Conclusion

The microorganisms exhibiting high invertase activity were yeasts isolated from sugarcane juice. The seven isolates of yeasts: *S. cerevisiae* MM2.1, *C. guilliermondii* 3M4.1, *C. humicolus* 5M2, *C. humicolus* 6M3.1, *S. cerevisiae* 6M4.1, *S. cerevisiae* 7M3.1.1 and *S. cerevisiae* 7M3.2.1, had high sucrose consumption rates. They had both extracellular and intracellur invertases. *S. cerevisiae* 6M4.1 exhibited the highest activity of both extracellular (0.95 U/mg) and intracellular (90.96 U/mg). This indicates that *S. cerevisiae* 6M4.1 has the potential for invertase production.

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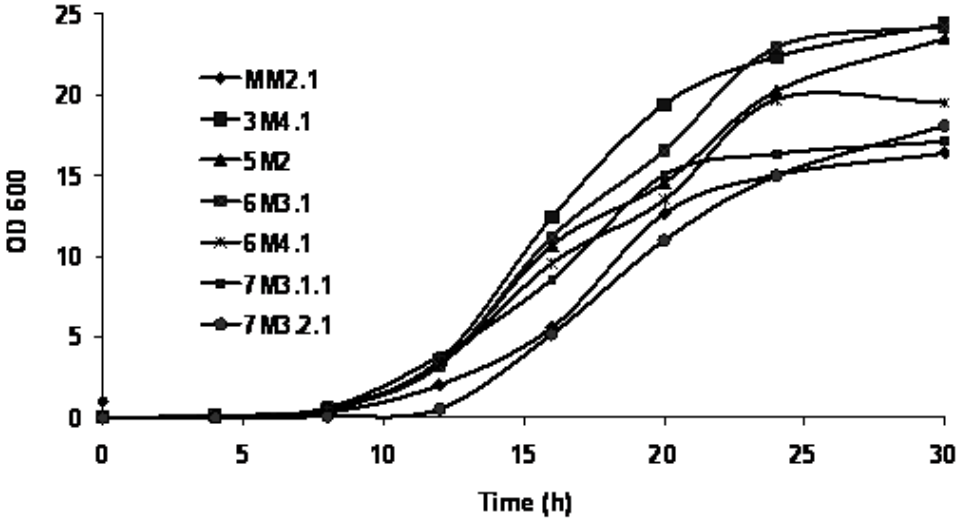

Figure 1. Growth curves of *S. cerevisiae* MM2.1, *C. guilliermondii* 3M4.1, *C. humicolus* 5M2 and 6M3.1, *S. cerevisiae* 6M4.1, 7M3.1.1 and 7M3.2.1 in complete medium containing 10% sucrose incubated by shaking at 160 rpm, 30 °C.

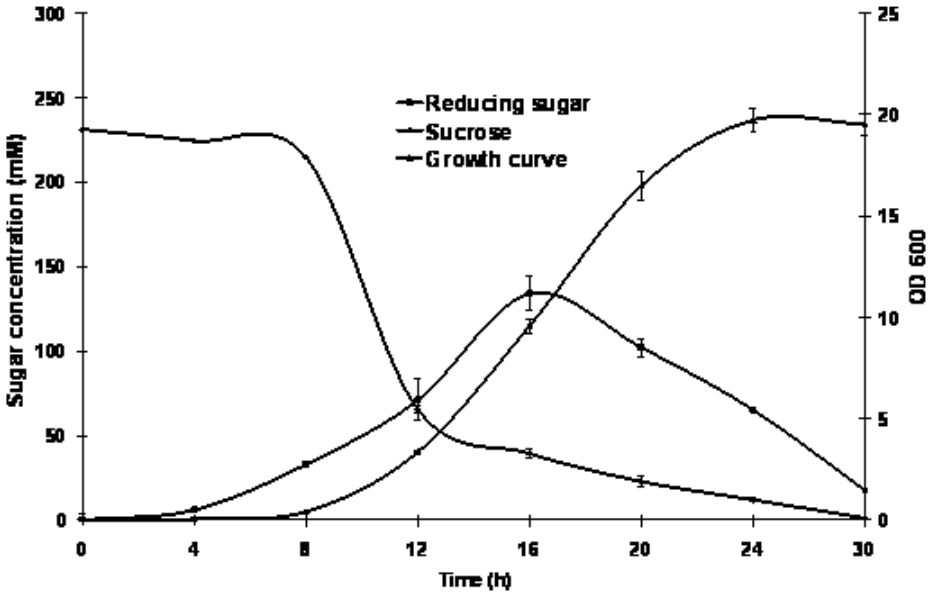

Figure 2. Growth and time course of sucrose concentration and reducing sugar liberated by *S. cerevisiae* 6M4.1 growing in CM liquid medium containing 10% sucrose incubated by shaking at 160 rpm, 30 °C.

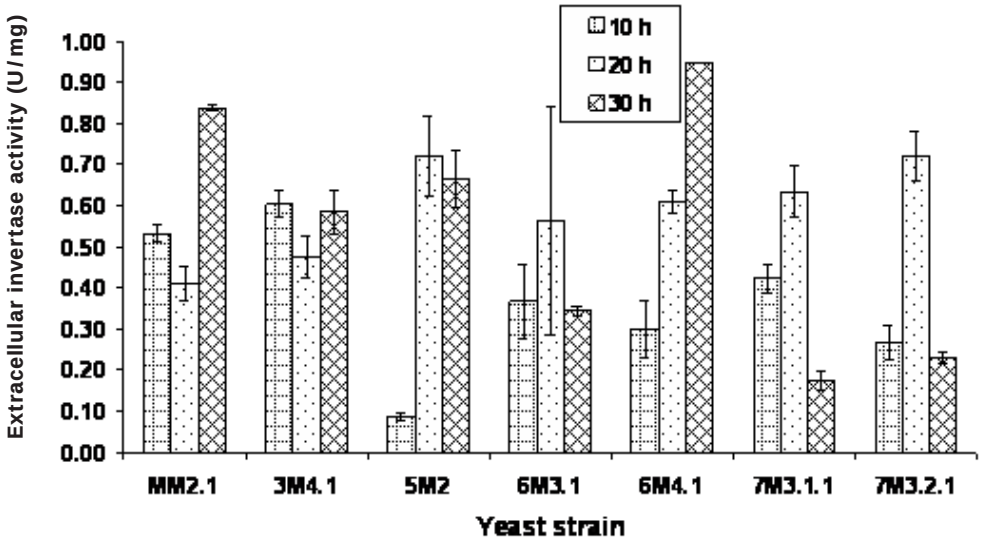

Figure 3. Extracellular invertase activity of 7 yeast strains in complete medium containing 10% sucrose cultured by shaking at 160 rpm, 30 °C for 10, 20 and 30 h.

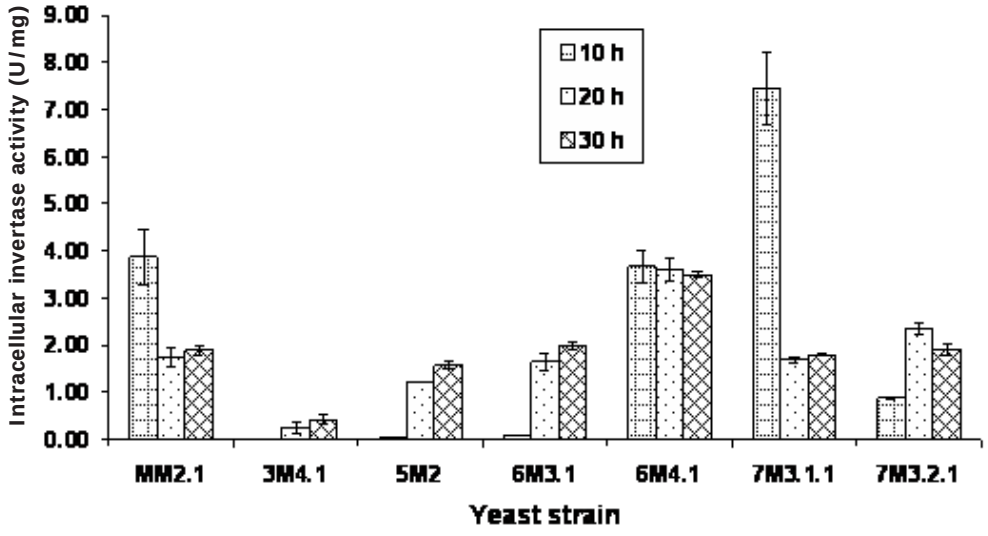

Figure 4. Intact intracellular invertase activity of 7 yeast strains in complete medium containing 10% sucrose cultured by shaking at 160 rpm, 30 °C for 10, 20 and 30 h.

Table 1. Activities of invertase from crude cell extract at stationary phase (30 h) of each yeast isolate with raffinose, melezitose and sucrose as substrates.

Isolate	Invertase activities (U/mg)		
	Raffinose	Melezitose	Sucrose
S. cerevisiae MM2.1	0.56 + 0.01	0	65.86 + 0.17
C. guilliermondii 3M4.1	0.41 + 0.03	0	33.56 + 0.65
C. humicolus 5M2	0.61 + 0.01	0	46.44 + 0.74
C. humicolus 6M3.1	0.75 + 0.03	0	56.38 + 1.42
S. cerevisiae 6M4.1	0.62 + 0.03	0	90.96 + 0.12
S. cerevisiae 7M3.1.1	0.48 + 0.01	0	56.09 + 2.25
S. cerevisiae 7M3.2.1	0.47 + 0.01	0	56.51 + 0.49

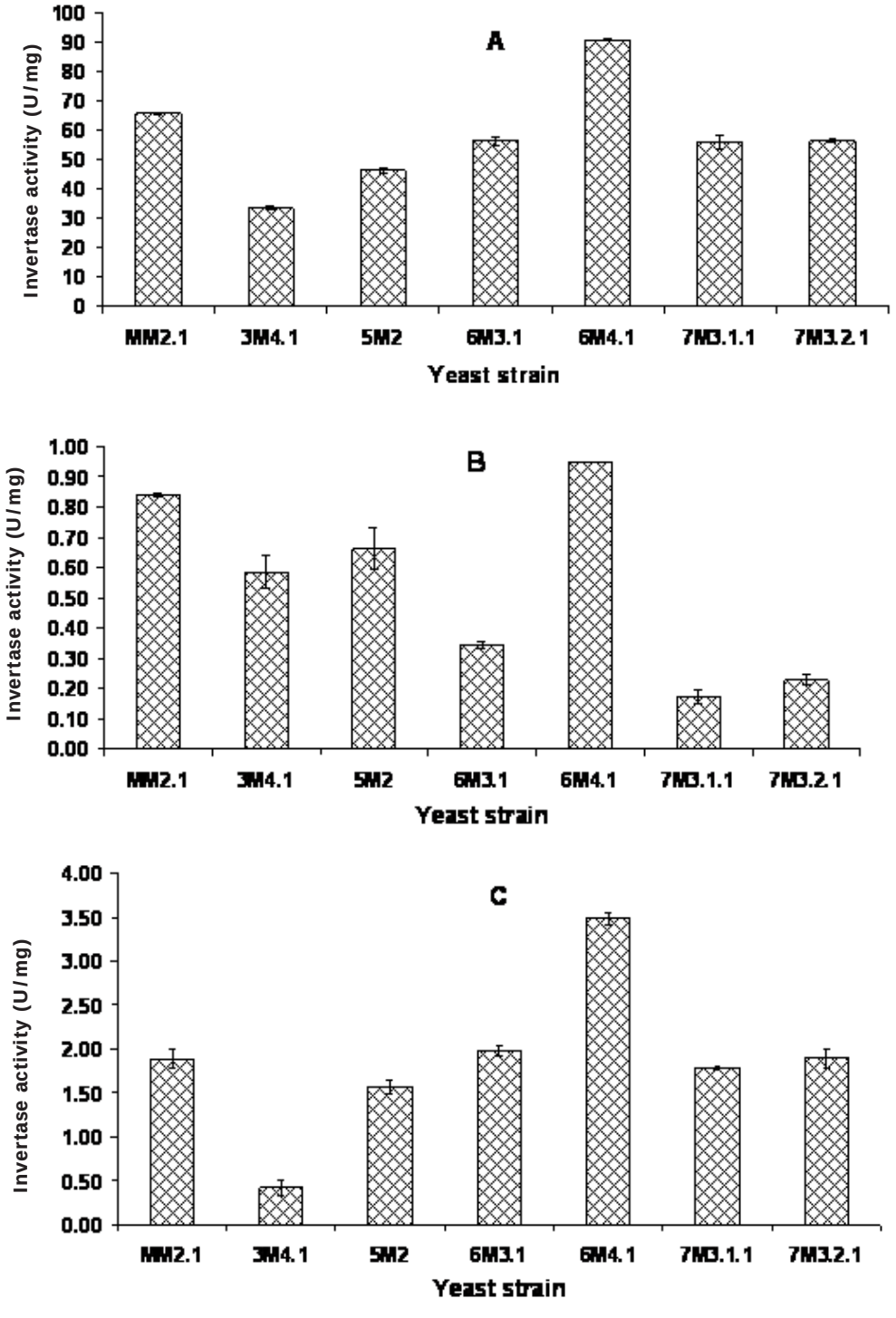

Figure 5. Invertase activity of 7 yeast isolates from complete medium containing 10% sucrose at stationary phase (30 h). A: Intracellular invertase activity from crude cell extract, B: Extracellular invertase activity from culture medium, and C: Intracellular invertase activity from intact cells.