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ORIGINAL PAPER

Partial purification of saccharifying and cell wall-hydrolyzing enzymes from malt in waste from beer fermentation broth

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Abstract A number of hydrolyzing enzymes that are secreted from malt during brewing, including cell wall-hydrolyzing, saccharide-hydrolyzing, protein-degrading, lipid-hydrolyzing, and polyphenol and thiol-hydrolyzing enzymes, are expected to exist in an active form in waste from beer fermentation broth (WBFB). In this study, the existence of these enzymes was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, after which enzyme extract was partially purified through a series of purification steps. The hydrolyzing enzyme activity was then measured under various conditions at each purification step using carboxymethyl cellulose as a substrate. The best hydrolyzing activities of partially purified enzymes were found at pH 4.5 and 50 °C in a citrate buffer system. The enzymes showed highest thermal stability at 30 °C when exposed for prolonged time. As the temperature increased gradually from 25 to 70 °C, yeast cells in the chemically defined medium with enzyme extract lost their cell wall and viability earlier than those without enzyme extract. Cell wall degradation and the release of cell matrix into the culture media at elevated temperature (45–70 °C) in the presence of enzyme extract were monitored through microscopic pictures. Saccharification enzymes from malt were relatively more active in the original WBFB than supernatant and diluted sediments. The presence of hydrolyzing enzymes from malt in WBFB is expected to play a role in bioethanol production using simultaneous saccharification and fermentation without the need for additional enzymes, nutrients, or microbial cells via a cell-free enzyme system.

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Introduction

Beer is produced by the saccharification of starch via the action of enzymes derived from malted cereal grains (most often barley and wheat), followed by fermentation of the resulting sugars by microbial-derived enzymes [1]. The discharged wet solid residue produced at the end of brewing, which is composed of starch, yeast cells, and liquid (mainly beer), is referred to as waste from beer fermentation broth (WBFB). WBFB is an inexpensive raw material with a high nutrient composition that is commonly used in the production of biologically important products including ethanol [2], microbial cellulose [3], and water-soluble oligosaccharides [4]. Because it is the waste of raw materials including starch, malt, etc., WBFB can be a rich source of a variety of enzymes. The existence of the saccharifying and starch-hydrolyzing enzymes and microbial species in WBFB has been reported [1, 5]. Moreover, direct elemental analysis of WBFB supernatant ensures the presence of certain macromolecules in WBFB [6].

Saccharifying enzymes from malt in WBFB randomly hydrolyze glycosidic bonding at multiple internal sites of the amorphous region of the polysaccharide [7]. The sole purpose of these enzymes is the production of simple sugars (mostly glucose) via the hydrolysis of complex polysaccharides into oligomers and then monomeric units [8]. The simple sugars produced via the saccharification process are utilized through a complex procedure in which the fermenting species produce ethanol. Being hydrolyzing agents in nature, these enzymes not only catalyze their

substrates (starch and other carbohydrate sources), but also affect the cell wall of fermenting microbial species [9]. Accordingly, the difference in the optimum temperature for enzyme activity (>50 °C) and microbial cell growth (<35 °C) is considered the major barrier for conducting simultaneous saccharification and fermentation [10]. Specifically, microbial species cannot survive at elevated temperatures, which affects the fermentation process. Accordingly, any process that can carry out fermentation utilizing fermentation enzymes at high temperature without considering the restriction of cell viability can significantly improve bio-ethanol production.

Sugars and other nutrients present in the liquid phase as a result of the activity of hydrolyzing enzymes must diffuse across the yeast cell wall to be metabolized by different fermentative enzymes into ethanol and other flavored compounds. The product formed inside the yeast cell ultimately diffuses into the media [11]. Thermal stress continuously increases membrane permeability and eventually causes cell death [12]. Upon cell death, the cell wall ruptures, resulting in release of the cell matrix, which consists of various enzymes, nutrients, and working machinery, into surrounding media. These compounds can then be utilized for the production of ethanol through usual microbial fermentation [13]. The development of the high-throughput in vitro glycoside hydrolase (HIGH) screening method has enabled the detection of glycoside hydrolase activity [14]. In the present study, we attempted to evaluate the existence of saccharifying and cell wall-hydrolyzing activities of various malt-derived enzymes in WBFB, their effect on yeast cell wall hydrolysis and their possible impact on bio-ethanol production.

Materials and methods

Waste from beer fermentation broth

Waste from beer fermentation broth was provided by the Aryana Hotel Beer Industry, Daegu, Korea. The WBFB was stored at 20 °C in closed cap containers for different periods of time. Microbial cells contained in WBFB were subsequently removed by filtration using Whatman® Schleicher & Schuell PVDF syringe filters with a size of 13 mm and 0.45 μm pore size before processing for enzyme extraction. Enzyme extraction was conducted using 1- and 3-month-old WBFB.

SDS-PAGE analysis

The molecular masses of hydrolyzing enzymes from malt in WBFB were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 15 % polyacrylamide gradient gel under reducing conditions using a Bio-Rad mini-gel apparatus [15]. Thermo Scientific PageRuler™ protein ladders with a range 10–170 kDa were used for calibration. The protein bands were visualized by staining with Coomassie brilliant blue R-250 (Sigma).

Partial purification

The original WBFB was shaken vigorously and the homogenate was then centrifuged at 12,000 rpm for 20 min, after which the supernatant was used as a crude extract. Solid ammonium sulfate (80 % saturation) was dissolved slowly in WBFB supernatant with continuous stirring. The proteinaceous part of the WBFB supernatant was precipitated by salting out with ammonium sulfate after overnight shaking incubation at 4 °C. The precipitates were obtained by centrifugation at a high speed of 12,500 rpm at 4 °C for 30 min. After centrifugation, the supernatant was discarded and the solid precipitate in the bottom was dissolved in citrate buffer (0.1 M, pH 4.5) at a ratio of 0.1 g/ml. The protein solution was then dialyzed against citrate buffer (0.1 M, pH 4.5) for 10 h at 4 °C with continuous stirring. To improve solute exchange, the dialysis buffer was replaced after each 2 h of incubation so that a new concentration gradient could be established. The dialyzed solution and ice cold acetone (100 %) were then mixed in equal ratios with continuous magnetic stirring. The precipitate that formed was collected through centrifugation at 12,000 rpm at 4 °C for 15 min and then dissolved in citrate buffer (0.1 M, pH 4.5). The enzyme solution was stored at −20 °C for further characterization as it is stable for >12 months in citrate buffer [16].

Determination of optimum pH

The optimum pH of the cell wall-hydrolyzing enzymes was determined using citrate buffers with different pH (3.0–6.0) at 50 °C for 30 min. The reaction mixtures were then used to measure the residual endoglucanase activity under standard assay conditions. Relative activity of the cell wall-hydrolyzing enzymes was measured on the basis that the activity of cell wall-hydrolyzing enzymes at pH 4.5 is 100 %.

Determination of optimum temperature and thermal stability of enzyme

The optimum temperature for the partially purified enzymes was determined by incubating the reaction mixtures at 30–80 °C for about 30 min. A standard cell wall-hydrolyzing enzymes assay was then carried out for each reaction mixture to determine optimum temperature on the basis of the activity result. Relative activity of the partially purified hydrolyzing enzymes was measured on the basis

that the activity of hydrolyzing enzymes at 50 °C is 100 %. Thermostability of the enzyme was measured by pre-incubating the enzyme solution at different temperatures (30–80 °C) for about 1 h. Samples of enzyme extracts were taken at different intervals and analyzed for residual activity through a standard assay [17]. Relative activity of the hydrolyzing enzymes was measured on the basis that the activity of hydrolyzing enzymes at 30 °C after different intervals is 100 %.

Enzyme assay

The activity of cell wall-hydrolyzing enzymes was determined as previously described [18]. Reaction mixtures containing 1 ml of enzyme extract, 1 % carboxymethyl cellulose (CMC) (substrate), and citrate buffer (pH 4.5) were mixed and incubated at 50 °C for 30 min. The quantity of reducing sugar produced as a result of enzymatic activity was then determined by the DNS assay protocol [17]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of glucose under experimental conditions.

Analytical methods

The concentration of glucose produced as the final product of polysaccharides hydrolysis by cell wall-hydrolyzing enzymes was determined using a glucose standard curve developed through the DNS (3,5-dinitrosalicylic acid) method [17]. Absorbance was measured at 575 nm using an UV-visible spectrophotometer (Model T60 U, Sunil Eyela Co., Ltd.). The total protein content in WBFB samples was determined after each purification step via Bradford assay using bovine serum albumin as a standard [19].

Microorganism

Yeast (*Saccharomyces cerevisiae*) was obtained from WBFB supernatant on chemically defined YM selective media. Briefly, an aliquot of WBFB was spread on agar plates of YM selective media prepared by dissolving 3 g/L glucose, 10 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, and 20 g/L agar in 1 L of distilled water. The culture plates were then incubated at 30 °C for 36 h, after which the colonies were counted for CFU determination. Morphological studies of yeast cells were carried out using a fluorescence microscope (JENMED, Germany).

Starch-hydrolyzing enzyme activity

Original WBFB, supernatant, and sediments were evaluated separately for saccharifying enzyme activity in the presence of starch as a substrate. In all three samples (6 ml), after dilution with 0.5 ml distilled water, 0.3 g of soluble starch was dissolved with continuous shaking. Following starch dissolution, the reaction mixtures were kept in a shaking incubator at 150 rpm for 2 days at 30, 50, and 60 °C. In another starch-hydrolyzing experiment, the original WBFB, supernatant, and sediments were incubated at 20 °C for 15 days. The glucose produced from soluble starch hydrolysis during incubation was determined using a glucose analyzer (Refractometer ATGO®, Japan) and glucose kit (GAHK20 Sigma-Aldrich, USA).

Results and discussion

The presence of hydrolyzing enzymes from malt in WBFB

Starch- and multiple hydrolyzing enzymes from malt play important roles in the process of beer production. Starch acts as a substrate for saccharifying enzymes derived from malt and are hydrolyzed into simple sugars that are further utilized by yeast cells to produce ethanol via the fermentation process. A number of hydrolyzing enzymes including cell wall-hydrolyzing enzymes, starch-degrading enzymes, protein-degrading enzymes, and lipid-hydrolyzing enzymes from malt are secreted during brewing. Detailed descriptions of the various enzymes that have reportedly been obtained from malt are shown in Table 1 [16, 20–39]. WBFB obtained at the end of the beer fermentation process was expected to contain some of these malt enzymes in active form [5]. To ensure the existence of various hydrolyzing enzymes in WBFB supernatant, we conducted SDS-PAGE analysis of 1- and 3-month-old WBFB supernatant (Fig. 1). The results revealed that several bands were produced from both 1- and 3-month-old WBFB samples. Comparison of the proteins bands with a molecular standard revealed the molecular weights of expected cell wall hydrolyzing enzymes and other malt-derived enzymes (Table 2). The bands produced from the 1-month-old WBFB samples ranged from 10 to 97 kDa, while those produced from the 3 month old WBFB samples ranged from 15 to 58 kDa. Comparison of the band positions with those of a standard and from previously conducted studies revealed that the one month old WBFB contained limit dextrinase [31], β-amaylase [30], carboxypeptidase [25], α-amaylase [29], endopeptidase [32], (1-3,1-4)-β-glucanase EI, EII [27], xylanase [23], endoglucanase [16], glucanase [28], and lipid transferase (LPT) [35]. The malt enzymes found in the 3-month-old WBFB were similar to those in the 1-month-old WBFB; however, some higher molecular mass peptides like limit dextrinase and lower molecular mass peptides were absent (<15 kDa) (Fig. 1).

Table 1 Major key enzymes of malt secreted in fermentation broth during brewing

Substrate	Class	Enzyme	Reference
Cell wall	1	Endoglucanase	[16, 20, 21]
		Xylanase	[20, 22, 23]
		Arabinofuranosidase	[20]
		Feruloyl esterase	[20]
		Acetoxylan esterase	[20]
		Carboxypeptidase	[24, 25]
		Exo-β-glucanase	[20]
		β-glucosidases	[20, 26]
		Glucanase	[27, 28]
Starch	2	α-amylase	[20, 22, 26, 29]
		β-amylase	[20, 22, 30]
		Limit dextrinase	[20, 26, 31]
		α-glucosidase	[20]
Protein	3	Endo-peptidase	[20, 32]
		Carboxypeptidase	[20, 22, 25, 33]
		Proteases	[22]
		Lipid transfer proteins	[34, 35]
Lipids	4	Lipase	[36]
		Lipoxygenase	[22, 37]
		Hydroperoxide lyase	[20]
		Hydroperoxide isomerase	[20]
		Hydrase	[20]
Phytin	5	Phytase	[38]
Polyphenol	6	Peroxidase	[39]
Thiols	7	Thiol oxidase	[20]

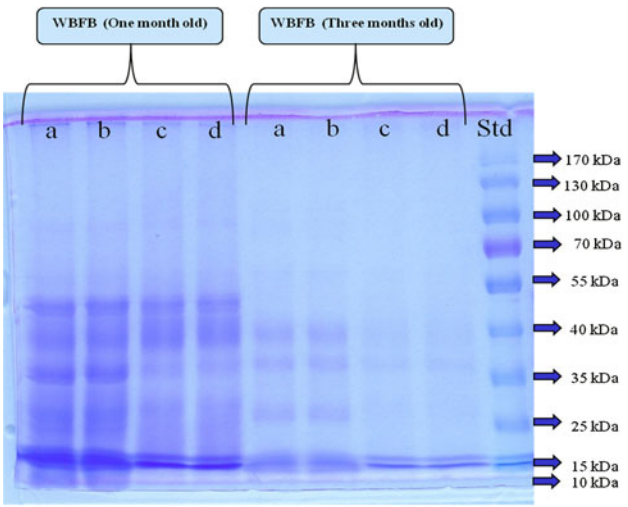

Fig. 1 Enzyme extract obtained after every purification step (**a** supernatant; **b** $(NH_4)_2SO_4$ saturation; **c** dialysis and **d** acetone precipitation) was analyzed by SDS-PAGE to determine the molecular weight of expected malt-derived hydrolyzing enzymes in WBFB

Table 2 Molecular weights of expected enzymes from malt in WBFB (1 and 3 month old) were determined after SDS-PAGE analysis using 15 % polyacrylamide gradient gel under denatured conditions

WBFB (kDa)		Expected enzymes	Class
1 month old	3 month old		
97		Limit dextrinase	2
58		β-amaylase	2
40–48	40–42	Carboxypeptidase III, α-amylase	1,2,3
37	37	Endopeptidase	3
25–30	28–29	(1-3,1-4)-β-glucanase EI, EII, Xylanase, Endoglucanase	1
15–20	15–20	Glucanase	1
9.5		Lipid transferase (LTP)	3

Less protein bands were recovered from the 3-month-old WBFB supernatant than the 1-month-old WBFB. These findings indicate that higher and lower molecular weight enzymes degraded with time; therefore, it is likely that the hydrolyzing activities of the 3-month-old WBFB supernatant are lower. The degradation of enzymes may have occurred due to variation in the WBFB composition with the passage of time and the decrease in pH as a result of the continuous production of acetic acid [2]. The half life of an enzyme, which is the time after which the enzyme loses 50 % of its original activity under standard conditions, is one of the most important parameters. The half life of an enzyme varies depending on conditions as enzymes are biodegraded or subjected to denaturation by others enzymes (protease), chemicals, and extreme pH and temperature stress [40]. Both decreasing pH and the presence of inhibitor favored the above statement that with the passage of time under unfavorable conditions, enzymes are subjected to denaturation due to their limited half life. Thus, the 3-month-old WBFB sample produced fewer enzyme bands than the 1-month-old WBFB.

Partial purification of hydrolyzing enzymes

Supernatant samples from both one month and 3-month-old WBFB were processed for partial purification of all malt-derived enzymes in WBFB. The purification of hydrolyzing enzymes in the WBFB supernatant was conducted through the following steps: filtration of supernatant, ammonium sulfate saturation, and concentration, dialysis against citrate buffer and precipitation in ice cold acetone (100 %) solution. After each purification step, the enzyme solution was analyzed by SDS-PAGE analysis (Fig. 1). The supernatant and ammonium sulfate precipitate samples of both 1- and 3-month-old WBFB were found to be rich in proteins and produce the maximum number of bands

Table 3 WBFB (1 and 3 month old) after every step of purification was processed for cell wall-hydrolyzing activity by incubation in the presence of 1 % CMC as substrate in citrate buffer at pH 4.5 and 50 °C for 30 min

WBFB	Sample	Total proteins (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)
3 month old	Supernatant	0.454	4.24	9.33
	$(NH_4)_2SO_4$ precipitates (80 %)	1.707	17.23	10.09
	Dialysis	1.209	15.67	12.95
	Acetone precipitation (100 %)	0.651	12.58	19.34
1 month old	Supernatant	1.064	12.63	11.86
	$(NH_4)_2SO_4$ precipitates (80 %)	2.337	36.37	15.56
	Dialysis	1.806	31.60	17.49
	Acetone precipitation (100 %)	1.567	29.58	18.88

among samples. The bands produced by the one month old WBFB sample ranged from 10 to 97 kDa; however, those produced by the 3-month-old WBFB ranged from 15 to 58 kDa. These findings demonstrate that various enzymes were removed during the purification process. Accordingly, this variation in band intensity with successive purification steps can be used to demonstrate the effect of each step on the hydrolyzing activities of various enzymes.

Cell wall-hydrolyzing enzymes activity

After confirming the presence of different hydrolyzing enzymes from malt via SDS-PAGE analysis, an enzyme assay for cell wall-hydrolyzing enzymes was carried out using CMC as a substrate. The cell wall-hydrolyzing enzyme activities after each purification step are given in Table 3. Specifically, Table 3 shows the total proteins, total activity and the specific activity of the cell wall-hydrolyzing enzymes obtained after successive purification steps at 50 °C and pH 4.5. The results revealed that the supernatant of both one month and three month old WBFB containing cell wall-hydrolyzing enzymes showed less catalytic activity (12.63 and 4.24 U/mL) than any other samples, regardless of the purification step. Solid ammonium sulfate has been widely applied to induce maximum protein precipitation from diluted samples while recovering only the proteinaceous portion of WBFB [41, 42]. Ammonium sulfate saturation and concentration by dissolving enzyme pellets in citrate buffer at 0.1 g/ml resulted in cell wall-hydrolyzing enzyme activity increasing from 12.63 and 4.24 U/mL to 36.37 and 17.23 U/mL in the 1- and 3-month-old WBFB, respectively. After dialysis and ice cold acetone treatment, the hydrolyzing activity of enzymes decreased to 31.60 and 29.58 U/mL in 1-month-old WBFB and 15.67 and 12.58 U/mL in 3-month-old WBFB, respectively. The increase in specific activity after each purification step ensured the purification of hydrolyzing enzymes from the WBFB supernatant and each purification step increased the efficiency of the active site of enzymes for the conversion of substrate into product.

Effect of pH on cell wall-hydrolyzing enzyme activity

pH is one of the most important factors influencing the reaction rate and reactivity of different enzymes. Similar to other reactants, enzymes are specifically active under a certain range of pH. Therefore, determining the optimum pH of a specific enzyme is of the utmost importance to achieve the maximum hydrolyzing activity. The optimum pH for cell wall-hydrolyzing enzymes was determined by measuring their hydrolysis activity in citrate buffer at 50 °C at pH ranging from 3.0 to 6.0. The selection of a citrate buffer system and 50 °C for the enzyme assay were based on previously reported data that have been used to determine the optimum cell wall-hydrolyzing enzyme activity [43, 44]. The results presented in Fig. 2 indicate that the catalytic activity of the enzyme extracts gradually increased as the pH of the buffer increased from 3.0 to 4.5. The maximum enzyme activity was obtained at pH 4.5; however, further increases in pH caused retardation and a gradual decrease in cell wall-hydrolyzing enzyme activity. The enzyme activity obtained at pH 4.5 was taken as 100 % and the activity obtained at the rest of the pH ranges was reported relative to this value. Initially, the activity of cell wall-hydrolyzing enzymes increased in the order of 16.07, 26.18, and 58.04 % at pH 3.0, 3.5, and 4.0, respectively. However, increases in pH above 4.5 resulted in loss of hydrolyzing activity to 26.03, 53.85, and 81.36 % at pH 5.0, 5.5, and 6.0, respectively. The results obtained for the optimum activity of cell wall-hydrolyzing enzymes were similar to those for endoglucanase (cell wall-hydrolyzing enzyme) extracted from *Zea mays* seedling cell walls [16] and *T. viride* [45]. The pH optima for certain cell wall-hydrolyzing enzymes obtained from different sources varied slightly from those in previous studies. For example, the optimum activity of *A. aculeatus* [46] and *T. aurantiacus*

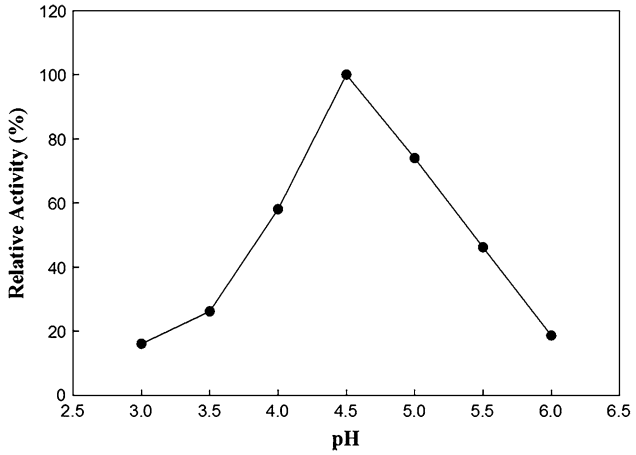

Fig. 2 Cell wall-hydrolyzing activity of enzyme extracts from WBFB. Samples were incubated in the presence of 1 % CMC as substrate in citrate buffer at different pH (3.0–6.0) and at 50 °C for 30 min. The activity of cell wall-hydrolyzing enzymes at pH 4.5 was taken as 100 %

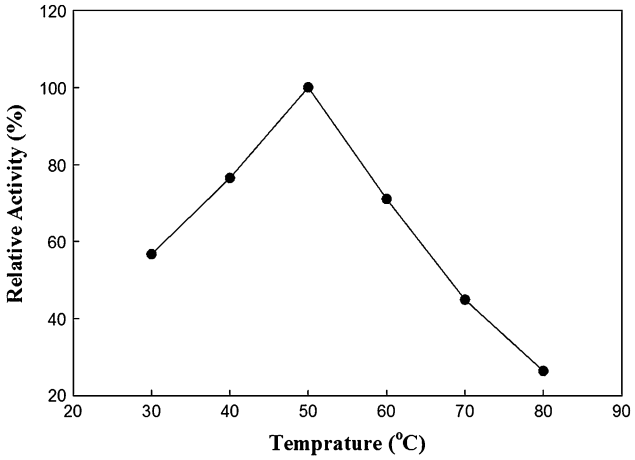

Fig. 3 Cell wall-hydrolyzing activity of enzyme extract from WBFB. Samples were incubated in the presence of 1 % CMC as substrate in citrate buffer at pH 4.5 and 30–80 °C for 30 min. The activity of cell wall-hydrolyzing enzymes at 50 °C was taken as 100 %

[47] endoglucanase occurs around pH 5.0. However, in most studies, the optimum activity occurs at pH values ranging from 4 to 5, which is in accordance with the results of the present study.

Effect of temperature on cell wall-hydrolyzing enzymes activity

Similar to pH, temperature also plays an important role in describing enzyme activity. The thermostability of an enzyme is a major factor determining the efficiency and level of certain enzyme-catalyzed reactions. Fermentation is generally carried out at 35 °C; however, most of the enzymes involved in the process show the optimum catalytic activity and thermal stability at 50 °C or higher [48, 49]. The optimum temperature for the activity of cell wall-hydrolyzing enzymes was determined by varying the temperature from 30 to 80 °C in a citrate buffer system while maintaining the pH at 4.5. As shown in Fig. 3, the activity of the cell wall-hydrolyzing enzymes increased in a linear fashion as the temperature increased to 50 °C, but further increases in temperature resulted in decreased activity. Therefore, the enzyme activity at 50 °C was taken as 100 % and the values at other temperatures were compared to this value. Initially, the cell wall-hydrolyzing activity increased by 56.73 and 76.48 % at 30 and 40 °C; however, increasing the temperature beyond 50 °C resulted in the hydrolyzing activity decreasing to 28.99, 55.1, and 73.62 % at 60, 70, and 80 °C, respectively. The results obtained for the optimum temperature are similar to those for endoglucanase (cell wall-hydrolyzing enzyme) obtained from the leaves of *Boscia senegalensis* [50]. Tao et al. [44] reported the same temperature optima for sugar cane bagasse-hydrolyzing enzymes from *Aspergillus glaucus* XC9.

Thermal stability of cell wall-hydrolyzing enzymes

Thermal stability is a desirable characteristic of an enzyme, especially in cases of industrial processing, where heat treatment may be inevitable. Therefore, the thermal stability of cell wall-hydrolyzing enzymes was evaluated by pre-incubating the reaction mixtures at different temperatures (30–80 °C) for 1 h. The results obtained for thermal stability are shown in Fig. 4. The catalytic activity was consistent at 30 °C, however, with further increases in temperature, the catalytic activity decreased. At temperatures above 50 °C, a sharp decline in activity was observed as the incubation time increased, with about 59 and 72 % of the enzyme activity being lost after 1 h of pre-thermal treatment at 60 and 70 °C, respectively. At 80 °C, 99.6 % of cell wall-hydrolyzing enzyme activity was lost after 1 h.

Effect of cell wall-hydrolyzing enzymes on yeast cell morphology and viability

One of the major challenges faced by free-living unicellular organisms such as yeast is their ability to adapt to stress conditions while maintaining their integrity and normal growth. Sudden and drastic changes in the external environment demolish the internal machinery of the cell, preventing normal growth. However, under certain stressed conditions, yeast cell surface proteins induce the signal transduction pathways of genes in different compartments specifying number of proteins translation involved in repair of intracellular damage [51]. Yeast cells have the ability to

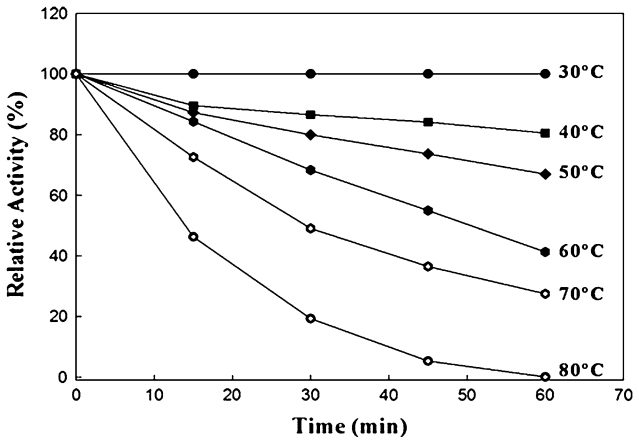

Fig. 4 Thermal stability of cell wall-hydrolyzing enzyme. Samples were preincubated at 30–80 °C for 1 h and the catalytic activity was measured under standard assay conditions. The activity of cell wall-hydrolyzing enzymes at 30 °C after different intervals was taken as 100 %

reprogram their genetic makeup; thus, each genomic expression is specific for environmental stress. Indeed, the expression of approximately 900 genes (14 % of total yeast genes) was found to be stereotypically altered from normal under stress conditions [52].

Yeast cells from WBFB supernatant were isolated and alterations in their morphology with gradual increases in temperature from 25 to 70 °C with and without the addition of WBFB cell wall-hydrolyzing enzymes extract were analyzed. Figure 5 shows a clear image of the yeast cell wall destruction caused by the synergistic action of thermal stress and cell wall-hydrolyzing enzymes. Yeast cells multiplied in a normal fashion for 45 min until the temperature reached 42 °C; however, longer incubation times and increased temperatures resulted in cell wall destruction by the synergistic action of thermal stress and cell wall-hydrolyzing enzymes activity. At 65 °C, almost all yeast cells lost their cell wall after 105 min of incubation, resulting in the cell constituents entering the surrounding medium. High thermal stress decreases cell viability by altering the mitochondria and increasing the membrane permeability, eventually resulting in cell death and cell lysate diffusion into the medium [12, 53]. However, the effects observed in the present study were not merely due to the high temperature stress, but also the presence of hydrolyzing enzymes. The effects can be more clearly understood by observing the CFU results (Table 4).

The CFU data revealed that the cell density increased as the incubation time and temperature increased up to 45 min and 42 °C, respectively. Most of yeast species showed optimum growth and fermentation at 30–35 °C. Yeast cells have a short generation time 1.25–2.0 h at 30 °C. However, gradual increases in temperature compel yeast cells to adapt to thermal stress. During the adaptation period (30–45 min), there is a very small increase in cell density when compared to the initial intervals (0–30 min). During the adaptation period, yeast cell growth decreased or ceased, which induced gene expression for a number of proteins involved in cell repairing and adaptation. However, once cells adapted to the stress maintained their integrity, multiplication continued as under normal conditions [54]. Contrary to abrupt chases in temperature, a gradual increase in temperature results in increased cell viability with 2–4 °C higher than the optimum are reached [55]. After 1 h, the temperature of the medium reached 48 °C, and the cell density started to decline, regardless of the presence of enzyme extract. When compared to chemically defined media without enzyme extract, the presence of enzyme extract resulted in lower yeast growth at all stages of analysis. Specifically, the live cell density was completely eradicated after 105 min in enzyme containing media, although a number of yeast colonies were still detected even after 2 h in bare thermally stressed media. Combined thermal stress and hydrolyzing activity of the WBFB cell wall-hydrolyzing enzymes caused the cell wall rupture earlier than when only thermal stress is applied; thus, the yeast cell culture lost viability earlier in the presence of enzyme extract. These findings indicate that hydrolyzing enzymes exert a destructive effect on the viability of yeast cells at high temperature.

Assessment of saccharifying enzymes from malt in WBFB

In our previous study, we evaluated the WBFB for the production of bioethanol without the addition of any enzymes, microbial cells, or carbohydrates in both shaking and static cultures [2]. Various saccharifying enzymes including α-amylase, β-amylase, and limit dextrinase from malt are added to the fermentation broth to initiate the starch saccharification process during brewing. However, some other enzymes including protein-hydrolyzing enzymes (e.g., endopeptidase and carboxypeptidase) and cell wall-hydrolyzing enzymes (e.g., endoglucanase and β-glucan solublase) are also added from malt [56, 57]. In this study, the existence of saccharifying enzymes activity in WBFB in the presence of substrate (starch) was determined at 30, 50, and 60 °C. The saccharification enzyme activity was evaluated using the original WBFB, filtered WBFB supernatant, and WBFB sediment with distilled water under shaking conditions. The initial glucose concentration in the original WBFB and supernatant was 5.5 %, while sediment diluted with distilled water contained 3.3 % glucose. The saccharification enzymes activity for the three aforementioned samples is given in Fig. 6a–c. After 24 h of incubation at 30 °C, the maximum glucose produced as a result of

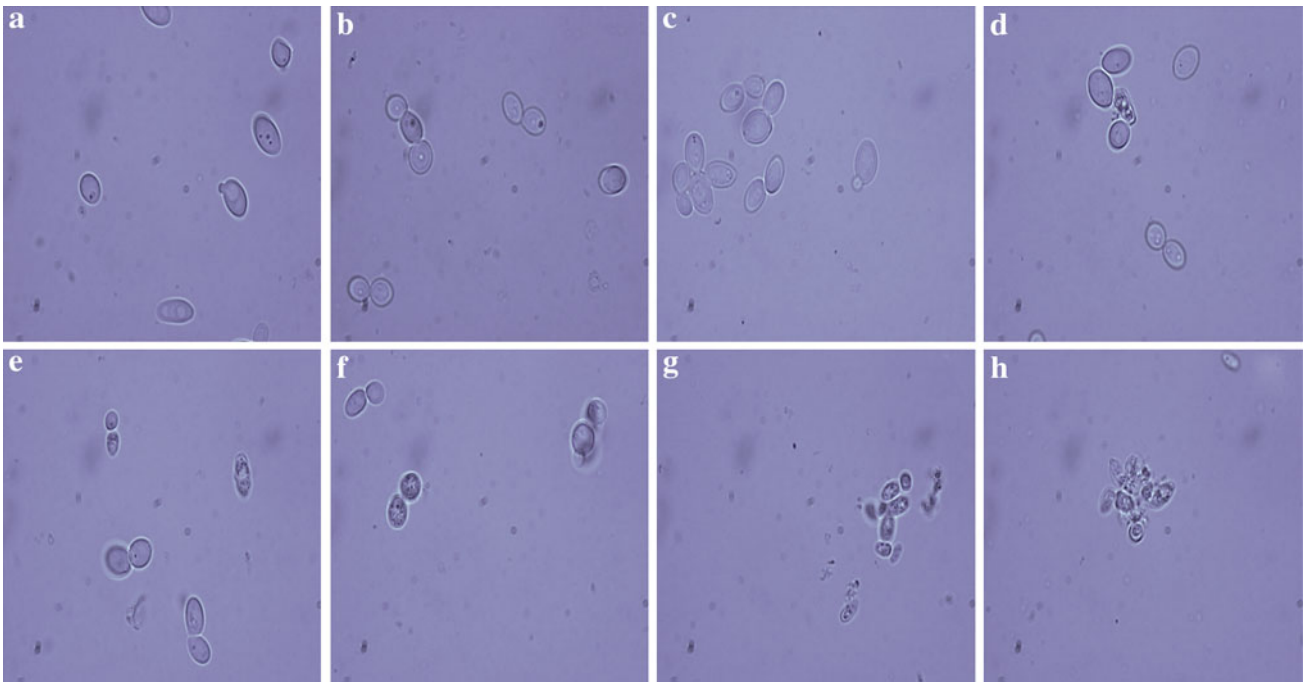

Fig. 5 Microscopic pictures of *S. cerevisiae* in chemically defined medium with enzyme extract taken at 25–70 °C after intervals of **a** 15; **b** 30; **c** 45; **d** 60; **e** 75; **f** 90; **g** 105; and **h** 120 min

Table 4 Variations in the CFU values of yeast cells in chemically defined medium with and without enzyme extract exposed to temperatures of 25–70 °C for about 2 h based on measurements at regular intervals of 15 min

Time (min)	Temperature (°C)	Average CFU of yeast in thermal stress only	Average CFU of yeast in thermal stress with enzyme extract
0	25	1.12×10^7	1.12×10^7
15	31	2.13×10^7	1.73×10^7
30	36.5	3.74×10^7	2.81×10^7
45	42	4.15×10^7	3.44×10^7
60	48	2.19×10^7	1.35×10^7
75	54	4.72×10^6	2.16×10^6
90	60	5.33×10^6	6.30×10^4
105	65	6.72×10^4	0
120	70	1.62×10^4	0

saccharifying enzymes activity in the original WBFB was 11.5 % (115 g/L), while it was 5.7 and 6.8 % in the supernatant and sediments with distilled water, respectively. The saccharification activity was enhanced as temperature increased to 50 °C, above which it decreased. At 50 and 60 °C, the original WBFB generated 12.8 and 11.9 % glucose. This was followed by sediments with distilled water (10.8 and 9.2 %, respectively) and supernatant (5.9 and 8.7 %, respectively). The result indicated the best saccharifying activities in original WBFB. The amount of saccharifying enzymes was higher in original WBFB that decreased in diluted sediments and WBFB supernatant. The highest saccharifying activity at 50 °C further partially supports the optimum temperature indicated in Fig. 3. All three samples were also evaluated for starch-hydrolyzing activity in the presence of soluble starch as substrate during incubation at 20 °C for 15 days. The hydrolyzing activities are shown in Fig. 6d. The maximum saccharification activity was obtained from the original WBFB (21.1 %) followed by diluted sediments (11.03 %) and supernatant (5.83 %). Based on the saccharification activity of the enzyme extract, the original WBFB is a rich source of hydrolyzing enzymes when compared to its supernatant and sediments. Being a rich source of hydrolyzing enzymes, the original WBFB can be used for higher bioethanol production as stated by Ha et al. [2].

Malt-derived enzymes involved in the saccharification process require the highest temperature for catalytic

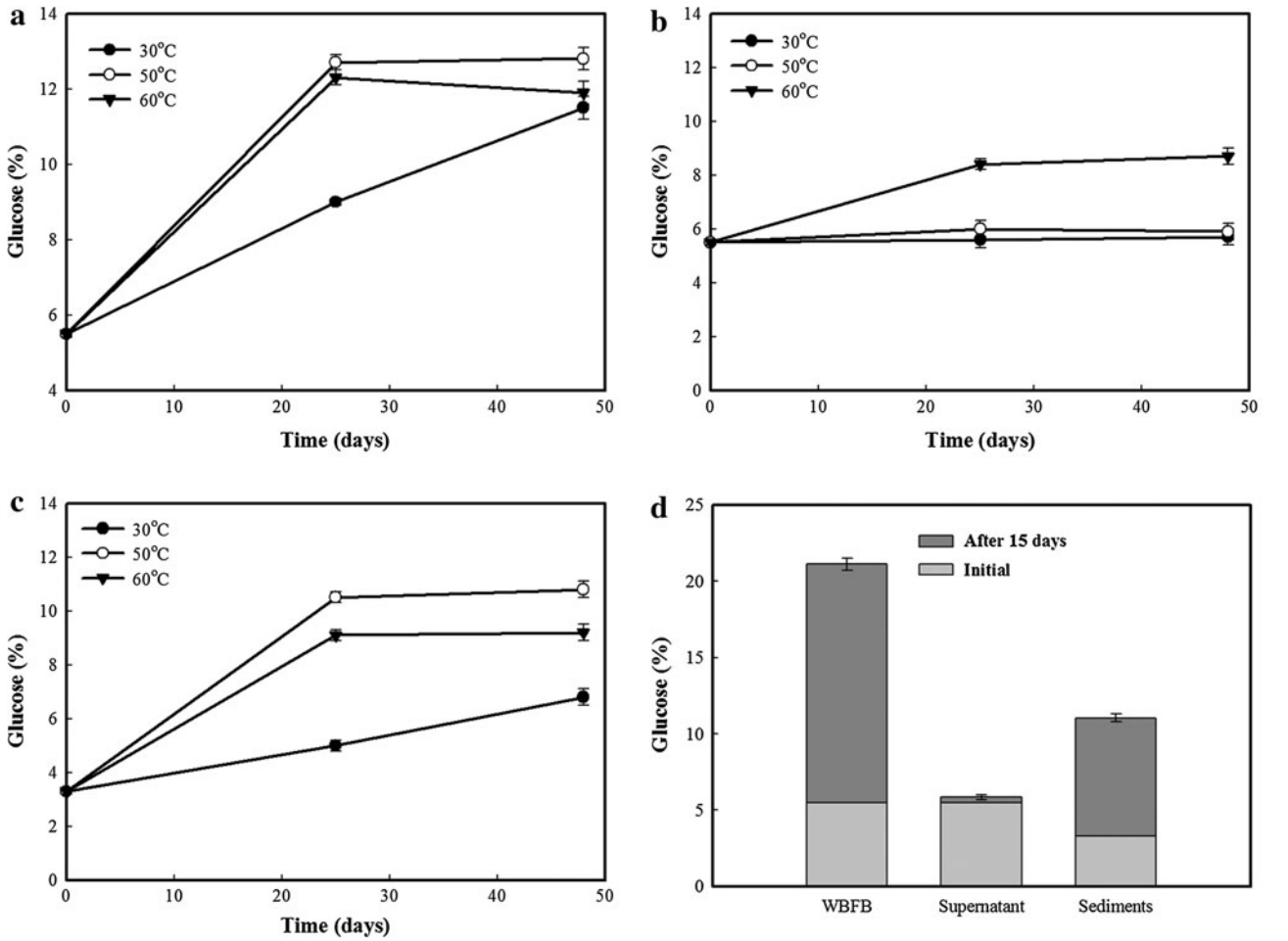

Fig. 6 Starch-hydrolyzing activity of enzyme extract from **a** original WBFB; **b** supernatant and **c** sediments with distilled water. Samples were incubated in the presence of soluble starch as substrate at 30, 50, and 60 °C for 2 days while shaking at 150 rpm and **d** all three samples were also evaluated for starch-hydrolyzing activity at 20 °C for 15 days

activity and even remain active at 60–90 °C [58]. However, yeast cells involved in the later fermentation of glucose to ethanol cannot survive these temperatures and lose their viability [59]. This causes the biggest hurdle in maintaining a combined saccharification and fermentation process for rapid and efficient bioethanol production. Several thermotolerant yeasts strains have been introduced to enable simultaneous saccharification and fermentation for maximum bio-ethanol production, but no successful increase in productivity has been achieved using this strategy [55]. The yeast cells present in WBFB are relatively weak as the culture has been used for repeated batches, making it more susceptible to autolysis by the synergistic action of cell wall-degrading enzymes and all other malt-derived hydrolyzing enzymes at high temperature when compared to fresh yeast culture produced in chemically defined medium.

Involvement of hydrolyzing enzymes in bioethanol production

The concept of a cell-free enzymes system was first introduced by Eduard Buchner [13], who claimed that free glucose molecules can be converted to ethanol by using only yeast cell extract composed of the enzyme zymase, which is responsible for that conversion. Cell-free enzyme systems enable the restriction of temperature required for cell viability and activity to be overcome, allowing the process to be carried out at higher temperatures required for saccharification [60]. It has also been reported that cell lysates released from microbial cells into the medium are composed of all machinery required for translation of polypeptides involved in the EMP pathway, ATP synthesis, and amino acid synthesis [14]. Therefore, in our case, the presence of cell wall-hydrolyzing enzymes activity along

with other malt-derived hydrolyzing enzymes in WBFB offer a great advantage to the overall procedure. Additionally, although thermal shocks can kill the yeast cells, the presence of cell wall-hydrolyzing enzymes hydrolyze the cell wall and release the internal matrix (nutrients, enzymes, and working machinery) to the culture media, enabling ethanol production through a cell-free enzyme system. The release of yeast internal materials to the media when treated at elevated temperature in the presence of cell wall-hydrolyzing enzymes can be seen in Fig. 5. After 45 min of incubation, the temperature reached 42 °C and cell wall hydrolysis started as a result of synergistic action of thermal stress and cell wall-hydrolyzing enzyme activity. Thus, almost all cells lost their cell wall at 65 °C after 105 min of incubation. Viability until a certain temperature and subsequent diffusion of the cell matrix into the culture media might govern the concept of cell-free ethanol production. The fermentation enzyme from yeast cells still might prove effective in the culture media and ethanol production.

Conclusion

The existence of various hydrolyzing enzymes in WBFB was confirmed through SDS-PAGE. Enzymes were partially purified through a series of purification processes and their hydrolyzing activities were investigated using CMC as a substrate. The hydrolyzing enzymes degraded the yeast cell walls and reduced their viability under gradually increasing temperature. The release of microbial cell matrix into the culture media may affect the bioethanol production process via a cell-free enzyme system. This concept might also be applicable to the production of bioethanol feedstock through cell-free biorefineries in future.

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References

1. Khattak WA, Ul-Islam M, Park JK (2012) Prospects of reusable endogenous hydrolyzing enzymes in bioethanol production by simultaneous saccharification and fermentation. Korean J Chem Eng 29(11):1467–1482
2. Ha JH, Shah N, Ul-Islam M, Park JK (2011) Potential of the waste from beer fermentation broth for bio-ethanol production without any additional enzyme, microbial cells and carbohydrates. Enzyme Microb Tech 49:298–304
3. Shehzad O, Khan S, Khan T, Park JK (2009) Production of bacterial cellulose in static conditions by a simple fed-batch cultivation strategy. Korean J Chem Eng 26:1689–1992
4. Khan T, Hyun SH, Park JK (2007) Production of glucuronan oligosaccharides using the waste of beer fermentation broth as a basal medium. Enzyme Microb Technol 42:89–92
5. Ha JH, Gang MK, Khan T, Park JK (2012) Evaluation of sediments of the waste from beer fermentation broth for bioethanol production. Korean J Chem Eng 29:1224–1231
6. Park JK, Hyun SH, Ahn WS (2006) Production of bacterial cellulose using waste of beer fermentation broth. Korean Chem Eng Res 44:52–57
7. Wood TM (1991) Fungal cellulases. In: Haigler CH et al (eds) Biosynthesis and biodegradation of cellulose. Macel Dekker Inc., New York
8. Han M, Kim Y, Kim Y, Chung B, Choi GW (2011) Bioethanol production from optimized pretreatment of cassava stem. Korean J Chem Eng 28:119–125
9. Lipke PN, Ovalle R (1998) Cell wall architecture in yeast: new structure and new challenges. J Bacteriol 180:3735–3740
10. Kádár Z, Szengyel Z, Réczey K (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Ind Crop Prod 20:103–110
11. Pilkington PH, Margaritis A, Mensour NA, Russell I (1998) Fundamentals of immobilized yeast cells for continuous beer fermentation: a review. J Inst Brew 104:19–31
12. Bischof JC, Padanilam J, Holmes WH, Ezzell RM, Lee RC, Tomkins RG, Yarmush ML, Toner M (1995) Dynamics of cell membrane permeability changes at supraphysiological temperatures. Biophys J 68:2608–2614
13. Buchner E (1897) Alkoholische Gährung ohne Hefezellen. Ber Dtsch Chem Ges 30(117–124):1110–1113
14. Kim TW, Chokhawala HA, Hess M, Dana CM, Baer Z, Sczyrba A, Rubin EM, Blanch HW, Clark DS (2011) High-throughput in vitro glycoside hydrolase (HIGH) screening for enzyme discovery. Angew Chem Int Ed 50:11215–11218
15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
16. Hatfield R, Nevins DJ (1986) Purification and properties of an endoglucanase isolated from the cell walls of *Zea mays* seedlings. Carbohyd Res 148:265–278
17. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
18. Mandels M, Hontz L, Nystrom J (1974) Enzymatic hydrolysis of waste cellulose. Biotechnol Bioeng 16:1471–1493
19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7:248–254
20. Bamforth CW (2009) Current perspectives on the role of enzymes in brewing. J Cereal Sci 50:353–357
21. Woodward JR, Fincher GB (1982) Purification and chemical properties of two β-glucan endohydrolases from germinating barley. Eur J Biochem 121:663–669
22. Aastrup S, Bautista N, Janser E, Dörreich K (2004) Choice of enzyme solution should determine choice of raw materials and process. In: Proceedings of the presentation given at world brewing conference, San Diego, USA
23. Chithra M, Muralikrishna G (2008) Characterization of purified xylanase from finger millet (*Eleusine coracana*-Indaf 15) malt. Eur Food Res Technol 227:587–597
24. Bamforth CW, Martin HL, Wainwright T (1979) A role for carboxypeptidase in the solubilization of barley β-glucan. J Inst Brew 85:334–338
25. Sørensen SB, Svendsen I, Breddam K (1989) Primary structure of carboxypeptidase III from malted barley. Carlsberg Res Commun 54:193–202
26. MacGregor AW (1987) α-Amylase, limit dextrinase and α-glucosidase enzymes in barley and malt. CRC CR Rev Biotechnol 5:117–128
27. Jamar C, du Jardin P, Fauconnier ML (2011) Cell wall polysaccharides hydrolysis of malting barley (*Hordeum vulgare* L.): a review. Biotechnol Agron Soc Environ 15:301–313

28. Trudel J, Grenier J, Asselin A (1998) Detection of enzymes active on various β-1,3-glucans after denaturing polyacrylamide gel electrophoresis. Electrophoresis 19:1788–1792
29. Kumar RS, Singh SA, Rao AG (2005) Thermal stability of alpha-amylase from malted jowar (*Sorghum bicolor*). J Agric Food Chem 53:6883–6888
30. Acquistucci R, Turfani V, Aureli G (2011) Amylase modiWcation induced by the germination process in organic barley. Eur Food Res Technol 232:583–590
31. Burton RA, Zhang XQ, Hrmova M, Fincher GB (1999) A single limit dextrinase gene is expressed both in the developing endosperm and in germinated grains of barley. Plant Physiol 119:859–871
32. Koehler SM, Ho THD (1990) A major gibberellic acid-induced barley aleurone cysteine proteinase which digests hordein. Plant Physiol 94:251–258
33. Sopanen T, Mikola J (1975) Purification and partial characterization of barley leucine aminopeptidase. Plant Physiol 55:809–814
34. Jones BL (2005) The endogenous endoproteinase inhibitors of barley and malt and their roles in malting and brewing. J Cereal Sci 42:271–280
35. Skriver K, Leah R, Mulleruri F, Olsen FL, Mundy J (1992) Structure and expression of the barley lipid transfer protein gene *Ltp 1*. Plant Mol Biol 18:585–589
36. Baxter ED (1984) Recognition of two lipases from barley and green malt. J Inst Brew 90:277–281
37. Doderer A, Kokkelink I, Van der Veen S, Valk BE, Douma AC (1991) Purification and characterization of lipoxygenase from germinating barley. In: Proceedings of the 23rd European Brewery convention congress, Lisbon, pp 109–116
38. Aehle W (2007) Enzymes in industry production and applications. Wiley-VCH, Weinhheim
39. Antrobus CJ, Large PJ, Bamforth CW (1997) Changes in the cationic isoenzymesof peroxidase during the malting of barley. I. Tissue location studies. J Inst Brewing 103:227–231
40. http://www.natureclean.com/bacteria-enzymes.htm
41. Chun CZ, Hur SB, Kim YT (1997) Purification and characterization of an endoglucanase from the marine rotifer, *Brachionus plicatilis*. Biochem Mol Biol Int 43:241–249
42. Farinas CS, Scarpelini LM, Miranda EA, Neto VB (2011) Evaluation of operational parameters on the precipitation of endoglucanase and xylanase produced by solid state fermentation of *Aspergillus niger*. Braz J Chem Eng 28:17–26
43. Kavitha S, Nagarajan P (2011) Fermentative production of endoglucanse—kinetics and modeling. Int J Eng Sci Tech 3:1894–1898
44. Tao YM, Zhu XZ, Huang JZ, Ma SJ, Wu XB, Long MN, Chen QX (2010) Purification and properties of endoglucanase from a sugar cane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9. J Agric Food Chem 58:6126–6130
45. Kim DW, Kim TS (1994) Purification of cellulase from *Trichoderma viride* and properties of its component enzymes. Bull Korean Chem Soc 15:719–724
46. Gajendra SN, Kaul P, Prakash V (2007) Purification and characterization of a new endoglucanase from *Aspergillus aculeatus*. J Agric Food Chem 55:7566–7572
47. Onsori H, Zamani MR, Motallebi M, Zarghami N (2005) Identification of over producer strain of endo-β-1,4-glucanase in *Aspergillus* species: characterization of crude carboxymethyl cellulose. African J Biotechnol 4:26–30
48. Karnchanatat A, Petsom A, Sangvanich P, Piapukiewb J, Whalley AJS, Reynolds CD, Gadd GM, Sihanonth P (2008) A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. Enzyme Microb Technol 42:404–413
49. Immanuel G, Bhagavath CMA, Raj PI, Esakkiraj P, Palavesam A (2007) Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. Int J Microbiol 3:1–20
50. Dicko MH, Leeuwen MJFSV, Traore AS, Hilhorst R, Beldman G (2001) Polysaccharide hydrolases from *Boscia senegalensis*: purification and characterization of endo-1,3-beta-glucanase. Appl Biochem Biotech 94:225–241
51. Gasch AP, Werner-Washburne M (2002) The genomics of yeast responses to environmental stress and starvation. Funct Integr Genomics 2:181–192
52. Gasch A, Spellman P, Kao C, Carmel-Harel O, Eisen M, Storz G, Botstein D, Brown P (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257
53. Swan TM, Watson K (1997) Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast. Can J Microbiol 43:70–77
54. Lu C, Brauer MJ, Botstein D (2009) Slow growth induces heat-shock resistance in normal and respiratory-deficient yeast. Mol Biol Cell 20:891–903
55. Banat IM, Nigam P, Singh D, Marchant R, McHale AP (1998) Review: ethanol production at elevated temperatures and alcohol concentrations: part I-yeasts in general. World J Microbiol Biotechnol 14:809–821
56. Halanych KM (1995) The phylogenetic position of the pterobranch Hemichordates based on 18S rDNA sequence data. Mol Phylogenet Evol 4:72–76
57. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
58. Han B, Kiers JL, Nout RM (1999) Solid-substrate fermentation of soybeans with *Rhizopus* spp.: comparison of discontinuous rotation with stationery bed fermentation. J Biosci Bioeng 88:205–209
59. Salvadó Z, Arroyo-López FN, Guillamón JM, Salazar G, Querol A, Barrio E (2011) Temperature adaptation markedly determines evolution within the genus *Saccharomyces*. Appl Environ Microbiol 77:2292–2302
60. Allain EJ (2007) Perspective Cell-free ethanol production: the future of fuel ethanol? Chem Technol Biotechnol 82:117–120