Studies on Isolation and Characterization of α-Amylases from Amylolytic Basidiomycete Yeasts

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(担子菌酵母由来 α–アミラーゼの単離とその特性に関する研究)

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CHAPTER 1

INTRODUCTION

Enzymes from bacterial and fungal sources have been increasingly applied in industrial sectors (40). Amylase is an enzyme that hydrolyses starch into its monomeric compounds, the smallest being glucose. The glyosidic bonds that hold the monomers together are broken down by the enzymes. This is a very common and essential reaction that takes place within various living organisms in order to generate or store energy. Therefore, amylase is a very prevalent enzyme produced biologically by various kinds of living beings. Amylases are one of the enzymes which can be derived from several sources such as plants, animals, bacteria and fungi. There have been great advances in the use of amylase in industrial sectors as well. A large portion of the enzyme market share is owned by amylase (14).

Large numbers of microbial amylase have completely replaced chemical hydrolysis of starch in starch processing industries (40). A wide range of industries such as food industries, garments, textiles and beverage industries along with medicinal and clinical chemistry use amylase to manufacture their products. This requires a constant production of amylase enzyme. Extraction of this huge quantity of amylase directly from nature is not feasible and hence various methods are being constantly established to develop the mass production of commercial amylase (2). Among the various types of amylase, the microbial amylase meets the industrial demands (38). Quite a large variety of microorganisms have been identified and chosen as the source of amylase production because of the availability and simplicity of the ways in which they yield amylase. Fungal amylases are used worldwide along with different strains of bacteria. Each strain of bacteria requires specific growth conditions and nutrients to produce amylase. Soil is a primary source of these bacteria which can be isolated and commercially grown in large numbers to produce a vast amount of amylase. In order to provide this, industries use fermentation shake flasks to grow bacteria (2). In addition, the amylases

that are extracted require optimum conditions to show greatest activity. This includes parameters such as temperature and pH (49). For this reason, it is important to find out the optimum conditions for amylase activity through research before it can be used for industrial purposes.

Amylase is a digestive enzyme classified as a saccharide (an enzyme that cleaved poly-saccharides). It is mainly a constituent of pancreatic juice and saliva, needed for the breakdown of long-chain carbohydrate (such as starch) into smaller units like disaccharides and trisaccharides. Although amylase can be derived from several sources such as plants, animals and microbes, the microbial amylase meets industrial needs and demands. Large numbers of microbial amylase have completely replaced chemical hydrolysis of starch in starch processing industries (40).

Nowadays the use of enzyme in industrial sector is increasing due to the increase of industries, especially in food, beverages, textile, leather and paper industries. Besides its uses in industry, it can also be used in treatment of industrial waste such as cellulase which is able to convert cellulose of wood and paper wastes to ethanol. One of the enzymes widely used in industrial sectors is alpha amylase. Screening may be described as the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest from among a large microbial population. The screening procedure saves a lot of time and labor without necessity of extensive studies to be carried out on each individual organism. Isolation and identification of such isolate is facilitated due to the presence of certain indicators, reagents/chemicals which aids in selection of the organism of interest (33).

Considering the above importance, the purpose of the present study is to isolate, screen and identify the amylase producing microorganism strains from Gifu area.

RESEARCH PURPOSE

The use of amylase enzyme is widespread globally in industrial sectors for the manufacture of a variety of products, including. However, scarcity of commercial amylase production in the country is a major problem for such industries. In order to eliminate the issue, it is necessary to isolate and identify a local amylase producing strain. Also, the amylase enzyme needs to be characterized in order to understand the proper parameters in which it shows best activity.

Therefore, this study is conducted with the aim to:

- Isolate potent amylase producing microorganism strains from Gifu Prefecture area
- Screen for the best producer of amylase by:
  - Observation of clear zone of starch hydrolysis in starch agar plate
  - Determination of amylase activity of crude amylase produced in submerged fermentation
- Identify the best isolate by:
  - Biochemical tests analysis, morphological and microscopic characteristics
  - Molecular identification using 28S rRNA gene sequencing and phylogenetic tree construction
- Characterize the crude amylase by determination of:
  - Optimum temperature • Optimum pH

LITERATURE REVIEW

Enzymes

An enzyme is a biological catalyst which alters the rate of chemical reactions usually by speeding it up. An enzyme is a protein molecule with a specific shape to accommodate reactant molecules. One enzyme molecule can catalyze 10 million reactions in a single second. Enzyme works at relatively low temperature (usually about 40ºC) thereby saving energy and saving money. The exact function of enzymes is determined by their structure, which is a combination of the sequence of amino acids from which they are build up. The sequence will result in a certain three-dimensional structure that may be stabilized by moieties other than amino acids that are added to the protein backbone. Their complete structure determines their function. Factors that modify enzyme structure will often also affect the function. (5). The term enzyme is a very familiar word to many. As defined, enzymes are biological compounds that speed up biochemical and biological reactions. Their role is to act as chemical catalysts which accelerate the time of these reactions both internal and external to an organism's cell (15). Hence, the term 'biocatalyst' has also been coined to define an enzyme. Without enzymes, it would be difficult to continue life since a huge number of biochemical reactions are required for an organism to function quickly and effectively. For this reason, enzymes are extremely important and valuable in order for an organism to live.

Uses of Enzymes in the Industries

Enzymes are used in the industries in many areas. They are starting to replace petroleum-based solvents used to make vegetable seed oil, replacing harsh acids used in the production of glucose products such as corn syrup, replacing sulfides used in the tanning

industry and many other aspects. Today, enzymes are helping industry to manufacture products used by society in a way that is less harmful to the environment. Amylases is use in the production of high fructose corn syrup by converting starch to sugars. Trypsin is used for primary food processing for babies because it can predigest baby foods. Papain is used for meat tendering (54). Amylase is used to split polysaccharides from malt during mash process. Proteases help to remove remaining protein from yeasts, which make beer clearer and easily filtrated. Glucose isomerase is used to convert glucose into fructose in production of high fructose syrups from starchy materials. Fungal enzymes are used to transform starch to glucose (25). Amylases are used to degrade starch to lower viscosity thereby aiding sizing and coating of paper. Xylanases are used to reduce bleach required for decolorizing. Cellulases are used to smooth fibers, enhance water drainage and to promote ink removal. Amylases are used in detergents for machine dish washing to remove resistant starch residues Cellulases are used in biological fabric conditioners. Alpha amylases are used to improve bread quality and to increase shelf life. Xylanases decrease the water absorption and thus reduces the amount of water needed in baking which leads to more stable dough. Proteinases are used to improve dough-handling properties. (25).

Starch

The main substrate that amylase works on is starch. It is a complex polysaccharide containing α-1,4-glycosidic bonds between its glucose monomers. This glycosidic bond is stable at high pH but breaks down at low pH (1). Starch is a carbohydrate and is a very common and useful source of energy for plants, animals, microorganisms and humans (1). Starch is naturally produced mostly by higher plants as a result of photosynthesis; hence it is considered as a renewable raw material (51). During dark periods, starch is stored in the plastids as a source

of respiration. Some algae also produce starch called phytoglycogen (1). Starch has been widely used in industries in order to provide textural properties to many foods. It has been used as thickeners, colloidal stabilizers, gelling and bulking agents and water retention agents in food and many industrial processes (27)

Two different glucose polymers make up a starch molecule. These are amylose and amylopectin. Amylose is made up of around 6,000 glucose molecules arranged in a linear fashion via α-1,4-glycosidic bonds. Around 20 to 30% of the starch molecule consists of amylose (1). Amylopectin on the other hand, is a branched molecule. It consists of a linear structure made up of α-1, 4-glycosidic bonds which is similar to amylose, but the branches are formed by α-1,6-glycosidic bonds. Amylopectin is considered as one of the largest molecules in nature since it is made up of around 2,000,000 glucose units. It is the major component of starch, making upto 75 to 80% of the molecule (1).

Types of Amylases

α-Amylase

α-Amylase (α-1,4-D-glucan-glucanohydrolase, EC 3.2.1.1) is an extracellular enzyme that is widely distributed in animals, plants, and microbes (40,28). As shown in Fig. 1, α-amylase catalyzes degradation of α-1,4-glucosidic linkage, but could not cleave α-1,6-linkages, and it breaks down long-chain saccharides by acting at random locations along the starch chain in an endo fashion (14, 28, 43).

In the starch degradation step, α-amylase has important roles for reducing viscosity and the starch chain molecular weight, and an increase in the number of starch chain molecules. Therefore, microbiol α-amylases are used in various industries such as producing isomerized

sugar, bakery applications, and textile de-sizing; it is also used in the paper industry and has other industrial uses.

α-Amylase is a glycoside hydrolase (GH), and GHs are classified into 128 families based on sequence similarity (7), and almost all α-amylases are classified into GH family 13, which is the largest of the GH families, although some of them belong to families 57 and 119 (7).

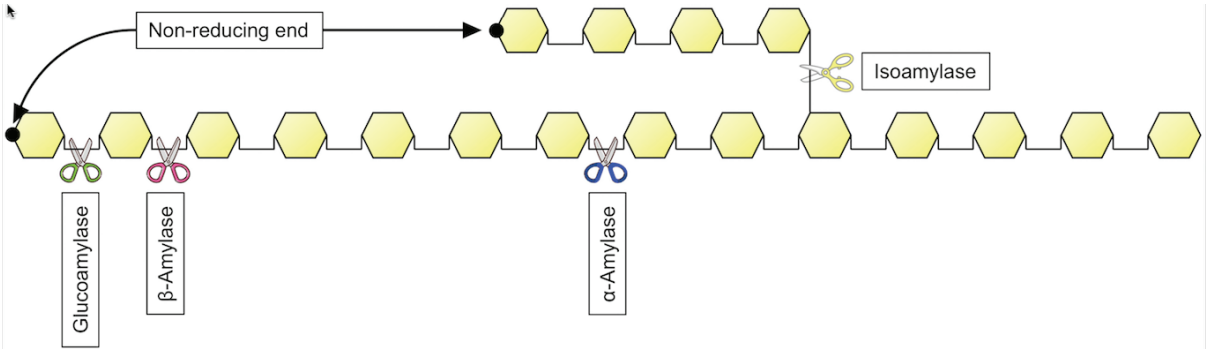

Figure 1. Cleavage sites of α-amylase, β-amylase, glucoamylase, and isoamylase on the amylose and amylopectin chain.

β-Amylase

β-Amylase (α-1,4-D-glucan maltohydrolase, EC 3.2.1.1) is an extracellular enzyme (11). β-Amylase is a major protein on the starchy endosperm of ungerminated barley seeds (16), and β-amylase is thought to be distributed only in higher plants, such as barley, soybean, and sweet potato (3; 34; 11), because β-amylase was found in malts in 1924. In 1974, however, new type of β-amylase was found in the Gram-positive bacteria Bacillus megaterium (17) and many β-amylases from various bacteria and fungi were subsequently reported (40; 45).

As shown in Fig. 2, β-amylase catalyzes degradation of α-1,4-glucosidic linkage, but could not cleave α-1,6-linkages, which is the same as α-amylase. β-Amylase, however, releases successive maltose units from the non-reducing ends from the starch chains (11). Therefore, in

industrial applications, β-amylase is used for fermentation in brewing and distilling industry and production of high maltose syrups.

β-Amylases are classified into GH family 14, which is the largest GH family (7).

Glucoamylase (γ-Amylase)

Glucoamylase (γ-amylase: 1,4-alpha-D-glucan-glucohydrolase, EC 3.2.1.3) is an extracellular enzyme that is widely distributed in animals and microbes such as bacteria and fungi (40). In animals, glucoamylase is located on the brush border of the small intestine, and in fungi, the enzyme is secreted into the medium.

As shown in Fig. 2, glucoamylase also catalyzes degradation of mainly α-1,4-glucosidic linkage, similar to α- and β-amylase, but it releases successive β-D-glucose units from the non-reducing ends from the starch chains (47; 50). Glucoamylase has wide diversities, i.e., glucoamylases form fungi, human intestine, and bacteria belong to different GH families, such as families 15, 31, and 97, respectively (7).

The major application of glucoamylase is the saccharification of partially processed starch/dextrin to glucose, which is an essential substrate for numerous fermentation processes and a range of food and beverage industries (48).

Isoamylase

Isoamylase (1,6-alpha-D-glucan-glucahydrolase, EC 3.2.1.68) is an extracellular enzyme that is produced by both plants and microorganisms. Isoamylase is a debranching enzyme of amylopectin, and it catalyzes the degradation of α-1,6-glucosidic branch linkage in amylopectin to yield amylose and oligosaccharides (52; 35). Isoamylases are classified into GH family 13 (7). Isoamylase is used primarily in the production of food ingredients from starch, such as glucose syrup, maltose, maltitol, trehalose, cyclodextrin, and resistant starch.

Microbial α-Amylases and Use of Microbial Amylase in Industries

Microbial amylases are rich in diversity, and several amylases with various properties have already been found and developed (40). Therefore, microbial amylases have high advantages to apply each industry compared with amylases from other sources because each industry requires specific amylases that have the suitable abilities for each application method. Microbial amylases are currently the main starch hydrolyzing enzymes that are used for starch saccharification and other applications, except for β-amylase from malt. However, among these industrial amylases, microbiol α-amylase is one of the most in-demand industrial enzymes in several industries.

The first amylase used industrially was fungal amylase in 1894 for the treatment of digestive disorders (27). Fungal and bacterial amylases are widely used in the industrial sectors (50). As it is very economical, fungal and bacterial amylase is the best choice of amylase to be used over other sources. Besides, the enzyme production rate is high, and the microorganisms can be genetically engineered for the desired quality and quantity of production of amylase. Amylase has a wide range of applications in food industries, textile mills and paper industries. In addition, it is used in the production of alcohol, detergents, bread, glucose and fructose syrup, fruit juices including fuel ethanol. Clinical, medical and analytical chemistry also uses bacterial α-amylases (15).

α-Amylase

Amylase was the first enzyme to be discovered and isolated by French chemists Anselme Payen and Jean-Franconis Peroz from germinating barley in 1833. In 1862, Alexander Danilewsky separated pancreatic amylase from trypsin (45). All amylase is glycosidic hydrolases and act on α-1,4-glycosidic bonds (24).

The enzyme amylase breaks down carbohydrate molecules into smaller products. Their main substrate is starch which is broken down into their smaller components such as dextrin, maltose, maltotriose, and glucose. The enzyme basically hydrolyses the α-1,4-glycosidic bonds that hold the glucose units together (15). Hence, amylase is also addressed as EC3 hydrolases. This class of enzymes breaks down molecules with the help of water. Some amylases belong to EC4 Lyases. Lyases remove groups to form double bonds or add groups to double bonds in substrates. Pectate lyase belong to EC5 Isomerases. These enzymes cause structural transformation of the same molecule by transferring groups from one position to another. Mutase belong to EC6 Ligases. Molecules are joined together via covalent bonds by these enzymes. These reactions require energy input in the form of co-factors called ATP. DNA ligase 7 'glycoside hydrolases'. Apart from starch hydrolysis, other forms of amylase known as transglycosylating enzymes, cause starch modification (49).

Types of amylase comes in two different types, endo-acting or endo-hydrolases, and exo-acting or exo-hyrolases (55). Endo-acting or endo-hydrolase type of amylase splits the interior glycosidic bonds of the amylose or amylopectin chain present in starch (1). Hence, the name endo-enzyme has been given to them. This type of amylase breaks down glycosidic bonds in a random manner (14). A well-known endo-amylase is the α-amylase. Most α-amylase enzymes require calcium ions for their activity.

α-Amylase have been derived from several fungi, yeast, bacteria and actinomycetes, however, enzymes from fungi and bacteria source have dominated applications in industrial sectors (40). α-Amylase may be derived from several bacteria, yeasts, and fungi. However, bacterial amylases are generally preferred over fungal amylases due to several characteristic advantages that it offers. Strains of *Bacillus* spp., mainly *Bacillus subtilis*, *B. megaterium* are employed (53).

α-Amylase is a key enzyme in the production of starch derivatives and also widely used in food, textile, paper, detergent, chemical, pharmaceutical and other industrial fields (4; 10; 28; 29).

α-Amylase is used in the food industry for the production of glucose syrups, crystalline glucose, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices (49).

α-Amylase is also used as an additive to remove starch-based dirt in the detergent industry. It is used in paper industry to reduce viscosity of starch for appropriate coating of paper. It is also used in the textile industry for warp sizing of textile fibers while it is used as digestive aid in pharmaceutical industry (49).

Bacterial α-amylase is widely used in pharmaceutical industry in various digestive aid preparations. Due to the presence of bacterial α-amylase, starch in the consumed food is better digested, it increases overall digestibility of the food. Digestive aid preparations made from α-amylase are used for treatment of patient whose digestive power is reduced due to illness. Many such a commercial formulation of digestive aid either as syrup or tablet are seen in many drug stores. Amylase is also used as a food additive. It is also used in clothing and dishwasher detergents to be dissolved starches from fabrics and dishes (9).

Microbial amylase is a very common enzyme found in nature. They are known to be produced most widely by microorganisms such as bacteria and fungi as compared to plants and animals (2). The enzymes are produced outside the cell, in order to carry out extracellular digestion (1). Fungi such as *Aspergillus niger*, *A. oryzae*, *Thermomyces lanuginosus* and *Penicillium expansum* are known to synthesize amylase (1). Among bacteria, the *Bacillus* spp. is well recognized as amylase producers. Some of these include *B. subtilis, B. licheniformis, B. amyloliquefaciens, B. stearothermophilus, B. cereus, B. polymyxa, B. coagulans* and *Lactobacillus plantarum* (1; 40). Other bacteria such as *Clostridium thermosulphurogenes,*

Proteus and Pseudomonas spp. are also acknowledged as amylase producers (1; 40). Bacillus is a common choice of source for industrial amylase production. Different strains of Bacillus have different optimal growth conditions and enzymatic production profile. Additionally, the genus produces amylase that is thermostable, has retention to extreme pH, osmolarity and high pressure. Other advantages of this bacterial enzyme production include use of short fermentation cycles, capacity to produce extracellular enzyme, simple cost effective 11 production, ecofriendly behavior, less handling hazard for workers, mass production and easy manipulation of bacterial genes (15). All these characteristics are ideal for industrial amylase production. Production and characterization of amylase is a hot topic of research. This is due to the vastness of applications of amylase in various sectors, especially in industries. Studies and experiments are being carried out on a large scale in order to detect and determine the easiest ways and optimal conditions to acquire amylase to meet the demands of product manufacture in the industrial world.

Starch

The main substrate that amylase works on is starch. It is a complex polysaccharide containing α-1,4-glycosidic bonds between its glucose monomers. This glycosidic bond is stable at high pH but breaks down at low pH (1). Starch is a carbohydrate and is a very common and useful source of energy for plants, animals, microorganisms and humans (1). Starch is naturally produced mostly by higher plants as a result of photosynthesis; hence it is considered as a renewable raw material (51). During dark periods, starch is stored in the plastids as a source of respiration. Some algae also produce starch called phytoglycogen (1). Starch has been widely used in industries in order to provide textural properties to many foods. It has been used as thickeners, colloidal stabilizers, gelling and bulking agents and water retention agents in food and many industrial processes (27)

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Starch hydrolyzing process using amylases

All living things use sets of amylases in the biological metabolism of carbohydrates such as starch and glycogen (41). In the first strep, branched starch, such as amylopectin, is debranched by isoamylase, yielding amylose. Amylose is then hydrolyzed into smaller oligosaccharides in an endo fashion by α-amylase (14; 29; 43). Finally, oligosaccharides are broken down into glucose or maltose by glucoamylase (or β-amylase), and organisms absorb glucose or maltose into the cell to metabolize them. This starch hydrolyzing process is called as “starch saccharification”.

Hydrolyzed starch has applications in several industries such as the food, beverage, pharmaceutical, textile, and detergent industries. Amylases can be used to form a variety of starch-hydrolyzed products with numerous physical and chemical qualities for these industries (39). In these industries, until the 19th century, starch saccharification was achieved by acid hydrolysis using dilute HCl because the understanding of the potential advantages of biological catalysts was limited. Because several types of microbiol amylases have since been found and developed, many advantages of enzymatic starch processing have been shown over chemical starch hydrolysis. These examples include the following: (1) enzymatic processing can be

performed at low temperatures compared with the chemical method; (2) it is not necessary to use a corrosion-resistant container for the reaction and the waste water is neutral pH and clean because the hydrolytic reaction of starch by enzymatic processing can be performed at around neutral conditions; (3) glucose yields of enzymatic processing are higher than the chemical method; and (4) chemical methods form unwanted colors and bitter taste compounds in hydrolysates. Therefore, today, starch saccharification achieved using an entirely enzyme-based process (22; 47)

CHAPTER 2

Material and Methods

Material

Handling of laboratory apparatus and glassware

All fresh glassware such as conical flasks, glass pipettes, beakers and glass rods were washed once with tap water and three times with distilled water. They were then air-dried prior use. All previously used glassware was autoclaved first at 121°C at 15 psi for 15 minutes in an autoclave machine and then used. Micropipette tips, eppendorfs and falcon tubes were autoclaved first and then used. All kinds of procedures were carried out by the student under the supervision of the lab officers and teaching assistants.

Use of media

Nutrient agar medium was used for short term preservation of bacterial cultures and for subculture purposes. Amy/YNB (yeast nitrogen broth) broth was used for the growth of bacterial culture before DNA extraction. Starch agar medium was used to screen amylase producing bacteria. A selective media was used during the fermentation of bacteria for amylase production in a shake flask.

Chemicals and Reagents

Coomasie Blue G-250, Sephadex G-25, DEAE-Sephadex, bacteriological peptone, dinitrosalicylic acid (DNS), sodium alginate were purchased from representatives of Sigma Chemical Co. Sodium dihydrogen phosphate, hydrogen disodium phosphate, sodium chloride, maltose, starch, glycerol, copper (II) tetraoxosulphate (VI), mercury(II) chloride, manganese(II) chloride, cobalt(II) chloride, zinc chloride, aluminium chloride, tris, glycine and ammonium sulphate are of analytical grade.

Equipment

UV-visible spectrophotometer, weighing balance, pH meter, incubator, micropipette, quartz curvettes, syringes (5ml, 10ml); ultracentrifuge were used. Most of these equipments were obtained in Biochemistry Department, Multi-user Science Research Laboratory and Center for Biotechnology and Research Training (CBRT).

Methods

Production of α-Amylase

The inoculum was prepared by the addition of about 5ml of sterile distilled water into the freshly grown nutrient agar slants. 0.5ml of the cell suspension was then inoculated into 100ml of sterilized fermentation medium and incubated at 40^0C for 48 hours. The composition of the fermentation medium in Amylase (starch), YNB media.

Bacterial strains and growth conditions

Stocked yeast strains in our laboratory were used in the screening of our amylolytic yeast strains. YNB medium with 1% (w/v) soluble starch (Amy/YNB) or 2% (w/v) glucose (Glc/YNB) as a carbon source was used for screening and cultivation of amylolytic yeast strains. Amy/YNB medium was also used in the screening of amylolytic yeast strains. These strains were incubated at 30°C for 24-48 hours under shaking or static conditions.

Extraction of α-amylase from the fermentation media

After incubation, the fermentation medium was harvested by centrifugation at 7,000×*g* for 30minutes at 4ºC. The cell-free supernatant was collected, and it was used to estimate amylase activity

Determination of Total Protein

The protein concentration was quantified according to the method described by Bradford, (1976). The assay is based on the principle that the maximum absorbance of an acidic solution of coomasie blue G-250 shift from 365nm to 595nm when binding of the dye to protein occurs. Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change.

Coomasie Brilliant Blue G-250 (25mg) was dissolved in 12.5ml 95% ethanol. To this solution, 25ml 85% (w/V) phosphoric acid was added. The resulting solution was diluted to a final volume of 250ml. Protein solution (0.1ml) was pipetted into test tubes. Three milliliters of protein reagent were added to the text tube and the contents mixed by shaking.

The absorbance at 595nm was measured after 5min and before 1hr against a reagent blank prepared from 0.1ml of the appropriate buffer and 3ml of the protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in the unknown samples.

α-Amylase assay

The enzyme assay was performed using the Rick and Stegbauer, (1974) method. The enzyme solution (0.5ml) was transferred to a test tube containing 0.5ml of 1.0% soluble starch solution. The mixture was incubated at 60ºC for 10min. Then 1.0ml of dinitrosalicylic acid reagent was added to each test tube. The tubes were placed in boiling water for 5min to stop the reaction and it was then cooled at room temperature. The contents of the tube were diluted up to 10ml with distilled water. The absorbance was then determined at 540 nm using a spectrophotometer and it was converted to micromole of maltose from the standard. One enzyme unit was taken

as being equivalent to that amount of enzyme which catalyses the hydrolysis of soluble starch into one micromole of maltose per milligram of protein per minute.

Phylogenetic analysis of isolated strains

Candidate strains of amylolytic yeast were identified by partial 28S rRNA D1/D2 sequencing. The partial 28S rRNA D1/D2 region was amplified with genomic DNA from candidate strains as a template by PCR, using an IROR primer (5'-ACCCGCTGAACTTAAGC-3') and an LR primer (5'-TACTACCACCAAGATCT-3').

Alignment and phylogenetic reconstructions were performed using the function "build" in the ETE3 software package v3.1.1[16] as implemented on GenomeNet (https://www.genome.jp/tools/ete/). The tree was constructed using FastTree with slow NNI and MLACC=3 (to make the maximum-likelihood NNIs more exhaustive)[17].

Detection of amylolytic activity on the plate

Amylolytic activity of the yeast strains was observed on Amy/YNB plates. Yeast cultures were incubated at 30ºC for 24-36 hrs. After incubation, iodine vapor was used to detect the amylase degradation halo on the plate[j]. The degradation of amylase was indicated by a clear zone indicating starch hydrolysis surrounding the colony.

Detection of amylase activity

Amylase activity was assessed by measuring the amount of sugar released according to the dinitrosalicylic acid (DNS) method[j]. A mixture of 1 ml of each enzyme solution and 1 ml of 1% soluble starch dissolved in 50 mM sodium phosphate buffer, pH 5.5, was incubated at 30°C for 10 min. The optimum pH of each amylase was determined using McIlvaine buffer[j]. The reaction was stopped by adding 1 ml of 3, 5-dinitro salicylic acid (DNS), which was followed

by boiling for 10 min. Distilled water was added to a final volume of 12 ml and the reducing sugar released was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol maltose equivalent per minute.

Active staining of amylase activity

Amylase zymograms of the extracellular fractions from strain GY16 were recorded. After native-PAGE, which was performed on 10% (w/v) polyacrylamide gel, loaded gel was incubated with 50 mM acetate buffer (pH 5.5) for 60 min, then incubated at 4ºC for 6 h in 50 mM acetate buffer (pH 5.5) containing 0.5% starch. After this incubation, the gel was incubated at 37ºC for 2 h, and bands with amylase activity were detected after staining with iodine solution (1 mM I_2 in 0.5 M KI).

Determination of the N-terminal amino acid sequence

N-terminal amino acid sequences were determined by the Edman degradation method using a peptide sequencer PPSQ30 (Shimazu Corp., Kyoto, Japan).

Identification of the *AMY1* gene on the genome of strain GY16

The *AMI1* gene on the genome of strain GY16 was amplified by the PCR method. The primers used for PCR amplification were: AMY1-Fw (5'-CAGTCTGGCTCCTTCTTCTTCCCG-3') AMY1-Rv (5'-AGGTGCTGCTCTTGGCCGTAGTAG-3'), AMY1-ATG (5'-ATGGCTCCTGTCCGCTCCCTAGC-3'), and AMY1-TAG (5'-CTAGGAGGACCACGTAAACTCG-3'). Obtained DNA fragments were sequenced according to the dideoxy cycle sequencing method with a BigDye Direct Cycle Sequencing Kit ver. 3.1 and a Model Applied Biosystems 3130 DNA Analyzer (Applied BioSystems,

Foster City, CA, USA). The Accession number of the *AMY1* gene on DDBJ/EMBL/GenBank databases is LC554832.

Determination of Optimum Temperature

The optimum temperature of the free and immobilized enzyme was determined in 50mM sodium phosphate buffer (pH 7.0) over a temperature range of 20 to 70ºC for 30min. 0.5ml and 0.5g of the free and immobilized enzyme was used for the test respectively

Determination of Optimum pH

The optimum pH for the free and immobilized enzyme was determined at 50ºC in 50mM citrate buffer (pH 4.0 to 5.0), 50mM sodium phosphate buffer (pH 6.0 to 7.0), 50mM Tris- HCl (pH 8.0 to 9.0) and 50mM Glycine-NaoH (pH 10.0 to 11.0). All pH values were adjusted at room temperature. 0.5ml and 0.5g of the free and immobilized enzyme was used for the test respectively.

Enzyme Assay

The method of determining the activity of the enzyme is known as enzyme assay. The component of an assay includes substrate, enzyme extract and diluents. The reaction mixture is incubated at a set of temperature and time to allowed reaction to take place. The reaction is stopped usually when the activity is still proportional to the time of reaction by changing the pH, application of heat, addition of protein denaturing agent or any other means. Once the reaction is stopped the amount of substrate remaining or product formed is determined. Several techniques can be used which include titrimetric, colorimeter, ultraviolet spectrophotometry and so on.

CHAPTER 3

Result

Screening of the yeast strains and their amylolytic activities

In previous reports, in order to isolate original *sake* yeast strains, we had screened 224 yeast strains with the ability to produce CO_2 during glucose static growth at 15ºC (36; 38). In the present study, drawing from stocks of these yeast strains that were stored in our laboratory, we screened all amylolytic yeast strains that were able to grow on Amy/YNB liquid medium to determine whether they had alcohol fermenting ability. None of our previously collected amylolytic yeast strains had this ability.

Accordingly, we set out to obtain and isolate new amylolytic yeast strains from natural environments in Gifu Prefecture, Japan. We were able to isolate six strains of amylolytic yeast, initially labeled as GY16, GY73, GY74, GY76, GY183 and GY184, all of which could grow on Amy/YNB plates under aerobic conditions (Fig. 2). Among these six strains, GY16 showed the best growth on plates, yet under static conditions its amylolytic growth was slightly weaker than that of the other strains (Fig. 3A). Strain GY73 showed high amylolytic growth under static conditions, although its growth was very slow (Fig. 2B). Nevertheless, none of the amylolytic yeast strains could produce any ethanol from starch during amylolytic growth (data not shown).

Next, we tried to visualize the amylolytic activity of these strains on plates. After each strain was allowed to grow on an Amy/YNB plate, the starch in the plate was stained with I_2. Clear zones indicating starch hydrolysis appeared around all growing yeast colonies. In particular, strain GY73 exhibited the largest clear zone among the strains (Fig. 2B).

Moreover, we compared amylase activity in the extracellular fractions of the amylolytic yeast strains. All strains grown on Amy/YNB medium in static culture exhibited

amylase activities, and strain GY73 showed the strongest amylase activity among these strains (Fig. 4).

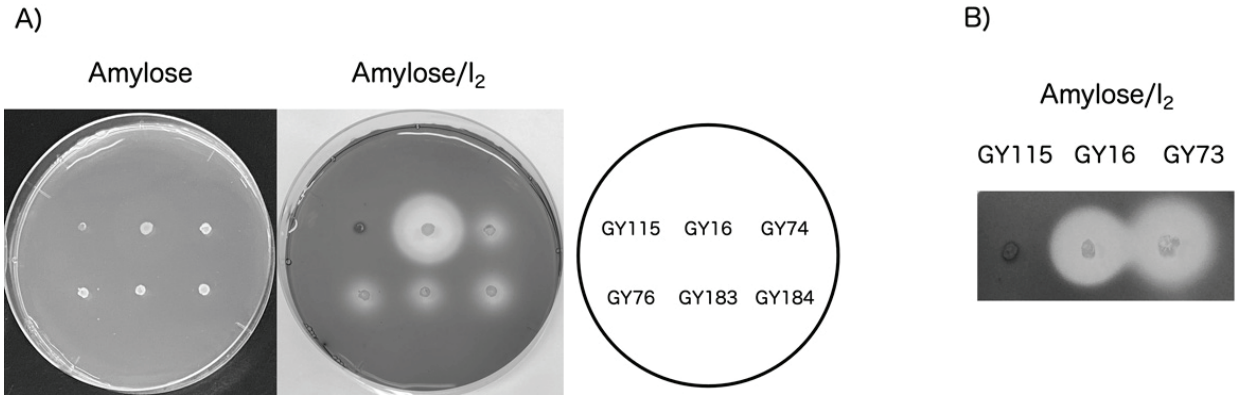

Fig. 2. Amylolytic growth and activity of the isolated yeast strains on Glc or Amy/YNB plates. After 72 h of growth at 28ºC, the plate was stained with iodine vapor. (A) GY strains in genuses *Saitozyma* and *Papiliotrema.* (B) Strain in genus *Ustilago* with GY16. *Saccharomyces cerevisiae* GY115 served as a negative control for amylolytic assay.

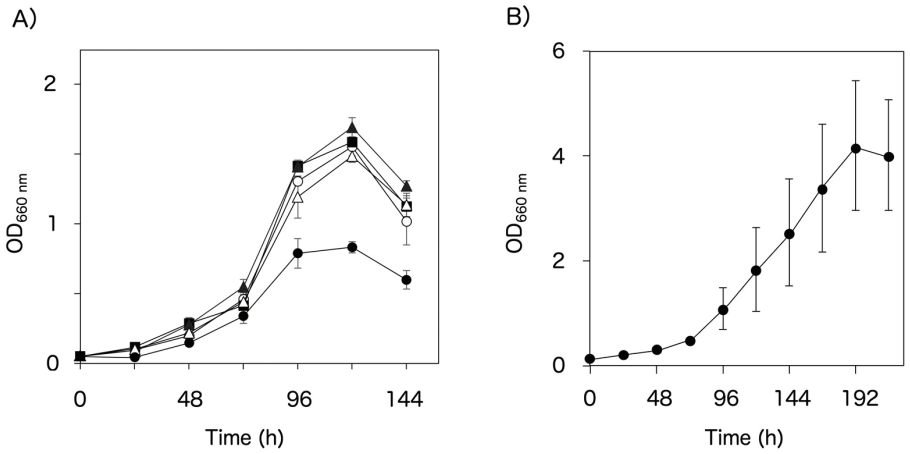

Fig. 3. Growth of amylolytic yeast strains on Amy/YNB medium in static culture. A) Strains in ordo *Tremellales*. Symbols: (●) GY16, (○) GY74, (■) GY76, (▲) GY183, and (△) GY184. B) Strains in ordo *Ustilaginales*. Symbols: (●) GY73. Error bars indicate the standard deviation of three replicates.

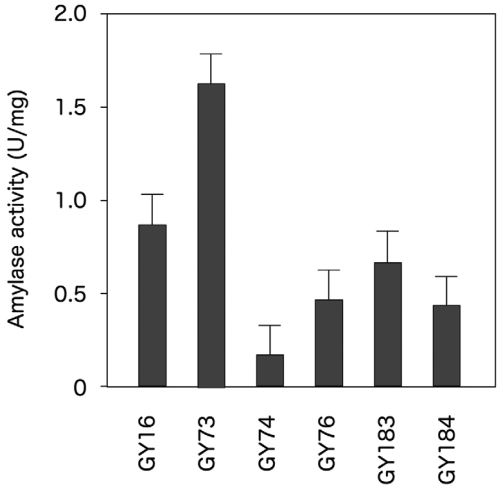

Fig. 4. Amylase activity of the amylolytic yeast strains. Error bars indicate the standard deviation of three replicates.

Identification of the amylolytic yeast strains

The amylolytic yeast strains were identified based on the sequences of their 28S rRNA D1/D2 regions. All yeast strains except for GY73 belonged to ordo *Tremellales* in *Basidiomycetes* (31) Strain GY16 showed the highest homology with strains of *Saitozyma flava* (syn. *Cryptococcus flavus*) (Fig. 5A). Strains GY74, GY76, GY183 and GY184 showed the highest homology with *Papiliotrema laurentii* CBS 10406^{T} (Fig. 4A). Therefore, we concluded that strain GY16 belongs to *Saitozyma flava*, and strains GY74, GY76, GY183 and GY184 belong to *P. laurentii.*

On the other hand, strain GY73 showed high homology with strains in ordo *Ustilaginales* in *Basidiomycetes*, and especially with the species *Ustilago filiformis*, *U. longissimi* and *U. davisii* (>99%) (Fig. 5B). Therefore, we concluded that strain GY73 belongs to genus *Ustilago,* but we could not identify the species of this strain; thus, strain GY73 is identified as *Ustilago* sp.

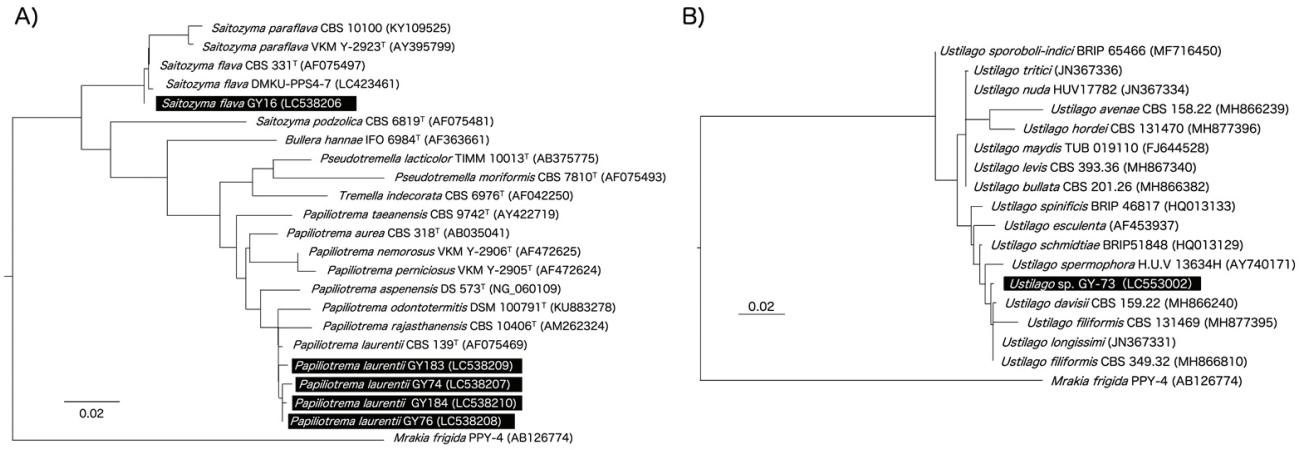

Fig. 5. Phylogenic relationships of isolated amylolytic yeast strains with related A) ordo *Tremellales* and B) ordo *Ustilaginales* yeast strains, based on the sequence of the 26S rDNA D1/D2 region.

Characterization of amylase from strains GY16 and GY73

Among the amylolytic yeast strains, we chose *S. flava* strain GY16 and *Ustilago* sp. strain GY73 as test strains for the characterization of amylase activity, because these two strains belonged to different orders, yet both showed relatively high amylase activity (Fig. 4).

First, using active staining in native-PAGE, we tried to generate zymogram patterns of proteins, which would show amylolytic activity in the extracellular fraction of strains GY16 and GY73 grown on Amy/YNB medium (Fig. 6A). Strains GY16 and GY73 each showed a single active band for amylase on native-PAGE. This indicated that both amylolytic yeast strains mainly secrete a single type of α-amylase into the medium.

Next, the components of the extracellular fractions of strains GY16 and GY73 grown on Amy/YNB medium were analyzed by SDS-PAGE (Fig. 6B). The extracellular fractions of GY16 and GY73 contained at least six and at least three types of proteins, respectively (Fig. 5B). Therefore, we identified the N-terminal amino acid sequences of these proteins. In the case of strain GY16, the N-terminal amino acid sequences of protein band A, which was ca. 66 kDa, and protein band D, which was ca. 35 kDa, were identified as

ANDIXQSPXVKNFN and GVQDMY, respectively, while bands B and C were not identified. Unfortunately, a FASTA search of our protein database revealed no proteins showing high homology with both N-terminal amino sequences. In the case of strain GY73, on the other hand, we identified the N-terminal amino acid sequences of the proteins in the extracellular fraction. The N-terminal amino acid sequence of band F was GTGSVGLTDQQ, which has high homology (77.8% identity and 100% similar) with the hypothetical protein encoded by UMAG_00064 in *U. maydis* strain 521 (27). This hypothetical protein is presumed to be extracellular aspartyl protease.

The optimal conditions for extracellular amylase activity in these two strains were then determined. In strains GY16 and GY73, the optimal temperatures for extracellular amylase activity were 55ºC and 60ºC, and the optimum pH were 5.0 and 5.5, respectively (Fig. 7).

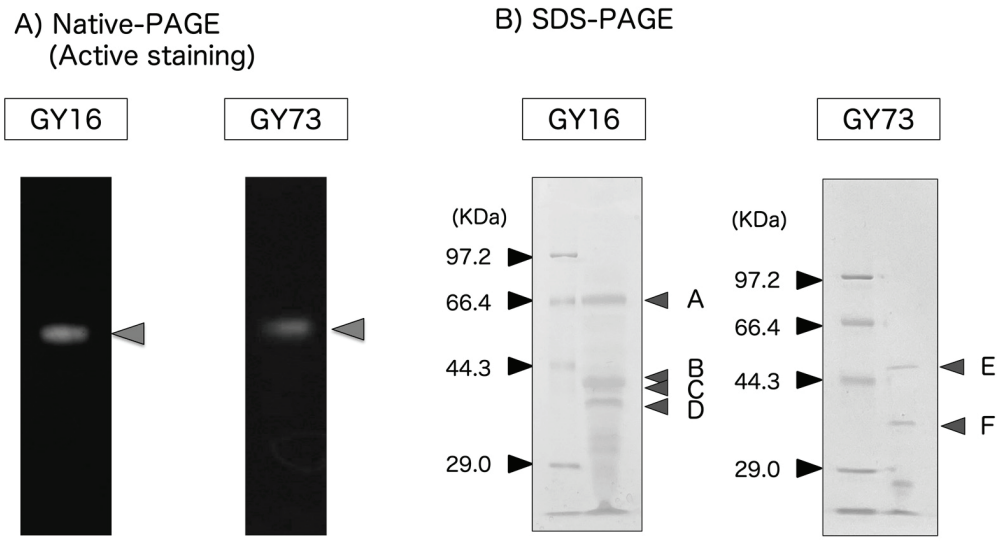

Fig. 6. Zymogram patters of (A) amylolytic enzymes on native-PAGE, and (B) extracellular proteins on SDS-PAGE for strains GY16 and GY73.

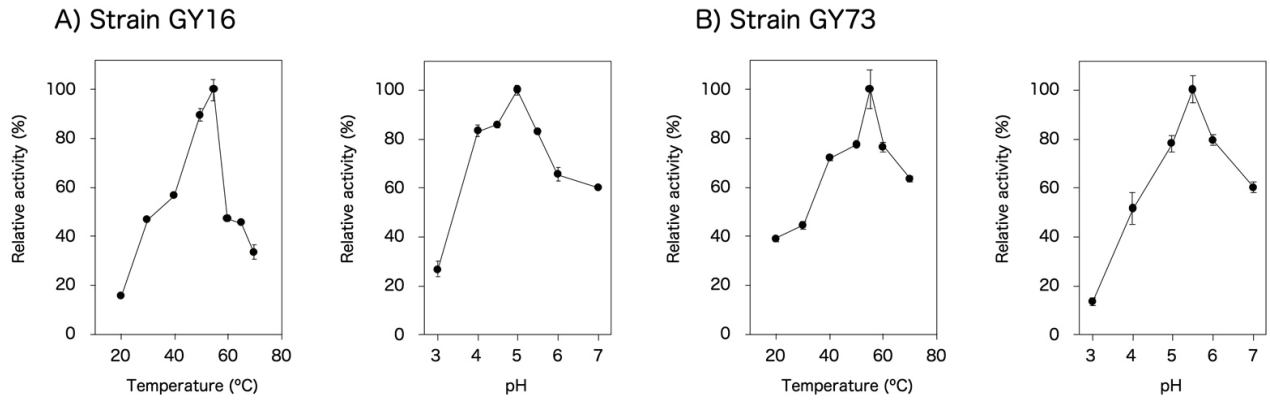

Fig. 7. Optimal temperatures and pH for extracellular amylolytic activity in (A) strain GY16 and (B) strain GY73. Error bars indicate the standard deviation of three replicates.

Identification of the *AMY1* gene on the genome of strain GY16

Finally, we cloned the partial *AMY1* gene encoding α-amylase on the genome of strain GY16. The partial *AMY1* gene was amplified according to the PCR method, using AMY1-Fw, AMY1-Rv, AMY1-ATG, and AMY1-TAG, which were designed from high-homology regions in the *AMY1* genes from other *S. flava* strains. The *AMY1* gene from strain GY16 contains three exons and two introns, like other *AMY1* genes (19). The deduced amino acid sequence of the *AMY1* gene from strain GY16 showed high homology to those of Amy1ps from other *S. flava* strains (>99.9% identity: Fig. 8). Amy1p from strain GY16 contained the α-amylase conserved regions I, II, III, and IV as well as a raw starch binding site (19) (Fig. 7). These results indicate that the *AMY1* gene on the genome of strain GY16 encodes a functional raw-starch-digesting α-amylase.

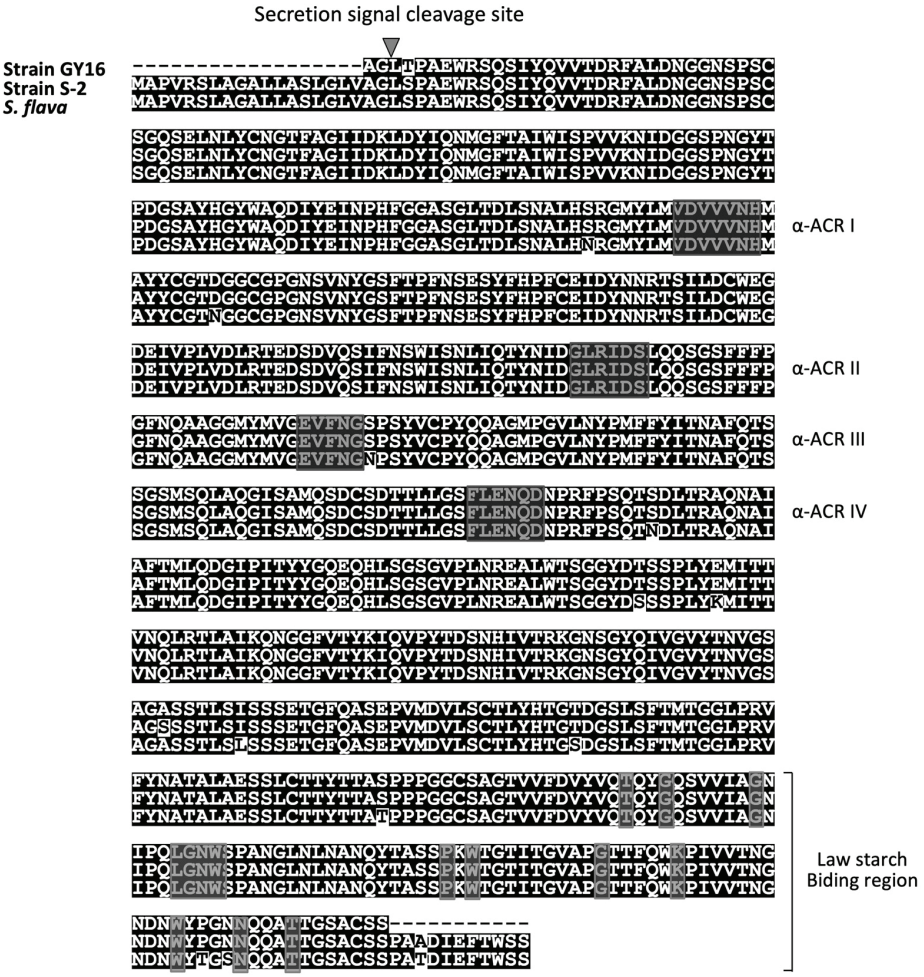

Fig. 8. Alignment of the deduced amino acid sequence of Amy1p from strain GY16 (Accession No. LC554832) with sequences of Amy1ps from strain S-2 (Accession No. BAA12010) and *S. flava* (Accession No. ABS76467). White letters indicate amino acid residues identical with F1d1p from strain GY16. α-ACR means "α-Amylase Conserved Region".

CHAPTER 4

Discussion

In this study, we isolated six amylolytic yeast strains belonging to *Basidiomycetes*. Although none of them had the ability to produce ethanol from starch, the isolated strains possessed sufficient amylolytic activity in the extracellular fractions.

Ustilago sp. strain GY73 and *S. flava* strain GY16 showed high amylolytic activity on Amy/YNB plates under aerobic conditions. This is the first report on amylases from genus *Ustilago*, although, like the others, strain GY73 did not have the capacity for ethanol production from starch. The amylases from strains GY73 and GY16 showed high optimal temperatures of 60ºC and 55ºC, respectively. In comparison, the optimal temperature for α-amylase from the *koji* mold *A. oryzae* is 50 to 55ºC(30) whereas that for α-amylase from the thermophilic fungi *Thermomyces lanuginosus* and *Myceliophthora thermophila* is likewise relatively high (60ºC) (23; 43). Therefore, strains GY73 and GY16 may produce fungal α-amylases that are thermophilic compared with other fungal α-amylases, although bacterial α-amylases show higher optimum temperature (around 90-100ºC) (20; 18).

Meanwhile, it has been reported that α-amylases from *S. flava* have the ability to digest raw starch (19). Moreover, the *AMY1* gene encoding α-amylase in the *S. flava* genome has already been cloned and then overexpressed in *Saccharomyces cerevisiae* (19, 12, 13). In this study, we found that strain GY16 showed a single active band for α-amylase, like other *S. flava* strains (12, 13), and cloned the partial *AMY1* gene from strain GY16. Given that the amino acid sequence of the *AMY1* gene from strain GY16 had high similarity (>99.9%) to those of *AMY1* genes from other *S. flava* strains, we propose that α-amylase from strain GY16 may also have the ability to directly digest raw starch at high temperatures. Using the *AMY1* gene from strain

GY16 and budding yeast strains, we predict that amylolytic alcohol-fermenting yeast strains can be bred in the near future.

As mentioned in this study, isolated six amylolytic yeast strains did not have special amylase activities, but the strains may have unique amylase activities. Moreover, *Ustilago* sp. strain GY73 may have extracellular aspartyl protease together with amylases (Fig. 5), and it was already reported that *P. laurentii* strains possessed xylanase, protease, and lipase activities, together with amylase activity in extracellular fraction (21). Therefore, we also consider that the isolated strains in this study also have a high potential as producers for the complex enzyme materials for food industry.

CHAPTER 5

SUMMARY AND CONCLUSION

SUMMARY

Amylases, which are starch-hydrolytic enzymes, have high potential for applications in several industries, such as food, textile, detergents, paper, pharmaceutical, chemical, and other many industries. Up to now, several types of microbial amylases have been developed, such as thermophilic, psychrophilic, acidophilic, alkaliphilic and other types of amylases, since industrial amylases are usually derived from bacteria, yeasts and fungi. However, depending on some processing steps, it may not be desirable for the starch to be completely degraded, or may require unique amylases, which show specific activities. In other words, these industries require not only highly active amylases, but also various types of amylases, which have suitable properties for each processing procedure.

In this study, the main aim was to isolate an amylase producing microbial strains, especially basidiomycete yeast strains, and characterization of their amylases.

Screening of the amylolytic yeast strains and their amylolytic activities

At first, I was able to isolate six strains of amylolytic yeast, initially labeled as GY16, GY73, GY74, GY76, GY183 and GY184, all of which could grow on Amy/YNB plates under aerobic conditions. Among these six strains, GY16 showed the best growth on plates, yet under static conditions its amylolytic growth was slightly weaker than that of the other strains. Strain GY73 showed high amylolytic growth under static conditions, although its growth was very slow. Next, I tried to visualize the amylolytic activity of these strains on plates. After each strain was allowed to grow on an Amy/YNB plate, the starch in the plate was stained with I2. Clear zones indicating starch hydrolysis appeared around all growing yeast colonies. In particular, strain GY73 exhibited the largest clear zone among the strains.

Identification of the amylolytic yeast strains

The amylolytic yeast strains were identified based on the sequences of their 28S rRNA D1/D2 regions. All yeast strains, except for GY73, belonged to ordo *Tremellales* in *Basidiomycetes*. Strain GY16 showed the highest homology with strains of *Saitozyma flava* (syn. *Cryptococcus flavus*). Strains GY74, GY76, GY183 and GY184 showed the highest homology with *Papiliotrema laurentii* CBS 10406T. Therefore, we concluded that strain GY16 belongs to *S. flava*, and strains GY74, GY76, GY183 and GY184 belong to *P. laurentii*.

On the other hand, strain GY73 showed high homology with strains in ordo *Ustilaginales* in *Basidiomycetes*; especially, species of *Ustilago filiformis*, *U. longissimi* and *U. davisii* showed high homology with strain GY73 (>99%). Therefore, we conclude that strain GY73 belongs to genus Ustilago, but could not identify species of the strain, thus strain GY73 is called as *Ustilago* sp.

Characterization of amylase from strains GY16 and GY73

I showed the optimal temperatures and pH of amylases from strains GY16 and GY73. The optimal temperatures for extracellular amylase activity were 55ºC and 60ºC, and the optimum pH were 5.0 and 5.5, respectively. Therefore, both amylases have thermophilic abilities among fungal amylases.

Next, I tried to show a zymogram patterns of proteins, which showed amylolytic activity, in the extracellular fraction of strains GY16 and GY73 grown on Amy/YNB medium, using active staining on native PAGE. Strains GY16 and GY73 each showed a single active band for amylase on native-PAGE. This indicated that both yeast strains mainly secrete a single type of α-amylase into the medium.

The components of the extracellular fractions of strains GY16 and GY73 grown on Amy/YNB medium were analyzed by SDS-PAGE. The extracellular fractions of GY16 and GY73 contained at least six and at least three types of proteins, respectively. Therefore, I identified the N-terminal amino acid sequences of these proteins. In the case of strain GY16, the N-terminal amino acid sequences of protein band, which was ca. 66 kDa, and band, which was ca. 35 kDa, were identified as ANDIXQSPXVKNFN and GVQDMY, respectively, while another bands were not identified. Unfortunately, a FASTA search of our protein database revealed no proteins showing high homology with both N-terminal amino sequences. In the case of strain GY73, on the other hand, we identified the N-terminal amino acid sequences of the proteins in the extracellular fraction. The N-terminal amino acid sequence of band F was GTGSVGLTDQQ, which has high homology (77.8% identity and 100% similar) with the hypothetical protein encoded by UMAG_00064 in U. maydis strain 521. This hypothetical protein is presumed to be extracellular aspartyl protease.

Identification of the *AMY1* gene on the genome of strain GY16

Finally, I cloned the partial *AMY1* gene encoding α-amylase on the genome of strain GY16. The partial *AMY1* gene was amplified according to the PCR method, using AMY1-Fw, AMY1-Rv, AMY1-ATG, and AMY1-TAG, which were designed from high-homology regions in the *AMY1* genes from other S. flava strains. The *AMY1* gene from strain GY16 contains three exons and two introns, like other *AMY1* genes. The deduced amino acid sequence of the AMY1 gene from strain GY16 showed high homology to those of Amy1ps from other S. flava strains (>99.9% identity). Amy1p from strain GY16 contained the α-amylase conserved regions I, II, III, and IV as well as a raw starch binding site). These results indicate that the *AMY1* gene on the genome of strain GY16 encodes a functional raw-starch-digesting α-amylase.

CONCLUSION

In this thesis, I isolated newly amylolytic basidiomycete yeast strains. They are also produced extracellular amylases. I consider that the isolated strains in this study have a high potential as producers for amylases for several industries.

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