

## Production and Characteristics of Yeast Dextranase from Soil

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### ARTICLE INFO

#### Article history:

Received August 2, 2017

Received in revised form November 12, 2018

Accepted November 30, 2018

#### KEYWORDS:

activity,  
dextranase,  
local isolate yeast,  
production,  
selection

### ABSTRACT

The existence of dextran in sugar cane juice is a major problem in the sugar industry, causing substantial losses. Treatment of dextran through enzymatic hydrolysis using dextranase is highly recommended as the most suitable method at this time because this is more effective and more economical. This study investigated the production and characterization of dextranase from local isolate yeast to degrade dextran on sugar cane juice. The selected yeast was identified on the basis of molecular identification. Dextranase was produced from the culture with the best carbon and nitrogen sources then was characterized. Application of enzyme was also evaluated. As a selected isolate, F4 had the closest relationship with *Pichia kudriavzevii*. The highest production of dextranase was induced by the supplementation of glucose and combination of yeast extract and peptone. The enzyme had optimum working condition at pH 7, temperature at 30°C and it is more stable at 4°C of storage temperature. The cation Na<sup>+</sup> played key role as co-factor while K<sup>+</sup> and Ca<sup>2+</sup> were detected as inhibitor of the enzyme. Dextranase from F4 isolate can hydrolyze dextran both in pure and in mixed dextran substrate, but with a lower hydrolysis rate.

### 1. Introduction

Sugar is one of important agricultural commodity in Indonesia, which people most need. Low domestic sugar production is led by a failure on controlling the delayed sugar cane milling period, which stimulates the establishment of dextran on sugar cane juice. Dextran is homoglycan of  $\alpha$ -D-glucopyranose molecules coupled primarily with  $\alpha$ -1.6 linkages and has branches on  $\alpha$ -1.2, 1.3, or 1.4 (Khalikova *et al.* 2005). According to Priyanka and Santosh (2011) dextran is a crucial problem along the sugar production process which can cause sugar cane juice detriment. Dextran-containing juice indicates low quantity of sucrose with high of viscosity therefore sugar crystallization process disturbed. Several methods to eliminate dextran such as physical and chemical methods have been applied in United States's sugar industry. These are very useful but are not yet technologically developed for their economical application in sugar cane process (Jimenez 2009). Therefore, the only

method applicable today in the sugar industry is enzymatic hydrolysis of dextran using dextranase (Purushe *et al.* 2012).

Dextranase is an enzyme that can hydrolyze primary linkage and also branching linkage on dextran. It can be synthesized by microorganisms such as fungi and bacteria (Esawy *et al.* 2012; Mahmoud *et al.* 2014). Recently, there are two dextranases from fungi that have been developed successfully and even commercialized. They are dextranase Novo from *Penicillium lilacinum* (Denmark) and dextranase Hutten DL-2 from *Chaetomium gracile* (Japan) (Khalikova *et al.* 2005). In Indonesia, both of dextranases are not yet well known even less applied in sugar industry due to the high cost. Additionally, a study of dextranase of yeast is still rare, whereas Indonesia with its megabiodiversity has the possibility for having species of yeast producing potential dextranase. High humidity soil provides a lot of microorganisms with various beneficial abilities such as dextran degrading yeast. The soil with this characteristic can be found in the reservoir edge. Hence, the aim of the present study was to produce

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and characterize dextranase from local isolate yeast to degrade dextran on sugar cane juice.

## 2. Materials and Methods

### 2.1. Procedures

#### 2.1.1. Selection of Dextranase Producing Yeast

Three local isolates yeasts (A4, C1, F4) origin Jatiluhur Dam soil, Purwakarta, Indonesia were cultivated on modified cultivation medium (1% dextran, 3% glucose, 0.5% bacteria peptone, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05% yeast extract) (Zohra *et al.* 2013). The isolates were incubated at 30°C for 24 hours, then selected based on qualitative and quantitative test. The qualitative test included measurement of hydrolytic zone around the colonies on semi solid dextran medium. Potential index of hydrolytic zone is a ratio between the diameter of hydrolytic zone with the diameter of the colony. After incubation, the plate was flooded with Congo Red solution to visualize hydrolytic zone or halo zone.

The quantitative test included measurement of crude enzyme activity on 1% dextran as a substrate. Dextranase activity was determined by measuring liberated reducing sugar during the enzyme-substrate reaction using DNS reagent (Miller 1959).

#### 2.1.2. Characterization and Molecular Identification of Selected Isolate

Characterization of F4 isolate included diameter size, color, elevation, edge, shape, and odor of the colonies on 1% dextran medium. Molecular identification was started with DNA extraction. Pure isolate was grown on solid dextran medium and incubated at 30°C for 24 hours. All growing colonies were collected and homogenized up using mortar. Yeast cells then centrifuged at 9,500 g for 1 minute. Selected yeast genomic DNA was extracted using peqGOLD Fungal DNA Mini Kit. The DNA of ITS was amplified using PCR machine. Fifty  $\mu\text{l}$  of PCR mix were created by composition: 25  $\mu\text{l}$  GoTaq Green Master Mix 2 (Promega, Madison, WA, USA), 2.5  $\mu\text{l}$  (100 pmol) for each primer: ITS5f (5'GGAAGTAAAAGTCGTAACAAGG-3') and ITS4r (5' TCCTCCGCTTATTGATATGC-3'), 0.3  $\mu\text{l}$  (64.4 ng/ $\mu\text{l}$ ) of DNA templates and 19.7  $\mu\text{l}$  nuclease free water. Polymerase chain reaction (PCR) was performed under the following conditions: pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 30

seconds, annealing at 55°C for 1 minute, elongation at 95°C for 2 minutes and final extension at 72°C for 5 minutes. PCR products were purified and sequenced by sending it to sequencing service company (Genetica Science). Sequences were analyzed using Bioedit program, then aligned with the ITS sequence database using BLAST-N program. Phylogenetic analysis was performed using MEGA 6 program and Neighbor Joining (NJ) with the bootstrap method 1000x (Tamura *et al.* 2013).

#### 2.1.3. Determination of Growth and Enzyme Activity of Selected Isolate

In this stage, the growth and enzyme activity curve were made simultaneously. A loop of cells after incubation for 24 hours was grown on 50 ml medium and incubated with shaking incubator until the optical density reached 0.6-0.8 nm to 600 nm of wavelength. After that, 2.2 ml culture was put on 217.8 ml new medium, then incubated for 18 hours. Cell turbidity and enzyme activity was measured every hour. Growth curve was constructed by standard curve of yeast. The amount of cell was determined by total plate count method on  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  of NaCl (0.85%) dilution.

#### 2.1.4. Selection of Carbon and Nitrogen Source for Dextranase Production

There were five media with different carbon source treatments, they are dextran, glucose, fructose, sucrose, and sugar cane juice medium. As a basal medium (1% dextran, 0.5% bacteria peptone, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05% yeast extract), dextran medium contained dextran as sole carbon source. Three other media involved basal media, which was supplemented 3% glucose, fructose, or sucrose, respectively. And the last medium was from sugar cane juice, which contained 3% of indigenous sugar. The yeast was grown in each medium, then incubated them on shaker incubation. After the production time was obtained, the crude enzyme was harvested for enzyme assay. The best carbon source was used for further investigation.

Various organic and inorganic nitrogen sources such as yeast extract, peptone, tryptone, ammonium sulphate, and urea were added in growth medium separately with 0.55% (w/v) of concentration. Besides of that, a medium contained 0.5% peptone and 0.05% yeast extract was also made up. The yeast, then was

inoculated into that media. The culture was incubated with shaking incubator until the optimum time for production was obtained. Crude enzyme then was harvested for enzyme assay. The best nitrogen source was used for further investigation.

### 2.1.5. Characterization of Dextranase

The activity of crude enzyme was examined on various pH solutions (4, 5, 6, 7, 8). The buffer solutions which were used were citric buffer for pH 4, citric-phosphate buffer for pH 5, phosphate buffer for pH 6, 7, and Tris-HCl buffer for pH 8. The effect of temperatures on dextranase activity was investigated by incubating the reaction mixtures at different temperatures (30°C, 40°C, and 50°C). To test the stability of the enzyme, crude enzyme was stored at 4°C and optimum temperature. Enzyme assay was conducted every hour under optimum pH and temperature conditions. Before culturing yeast, four kinds of cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>) from NaCl, KCl, CaCl<sub>2</sub>, MgCl<sub>2</sub> were added on growth medium separately until 1 mM and 5 mM of final concentration. Then, the crude enzyme then was harvested for enzyme assay.

### 2.1.6. Application of Dextranase to Degrade Dextran

5 ml of crude enzyme was mixed with 100 ml pure dextran substrate medium (1% b/v) and another one was mixed with 100 ml sugar cane juice (54.9 Brix) then incubated for 6 hours. Every hour 2 ml samples were taken up to measure the quantity of reducing sugar using DNS reagent (Miller 1959).

## 3. Results

### 3.1. Selection of Dextranase Producing Yeast

Three dextranase producing yeasts were grown on medium containing dextran and after 24 hours of incubation, these isolates were selected on the basis of hydrolytic zone of dextran hydrolysis. The hydrolytic zone was displayed by all isolates, but F4 isolate had the largest one with 1.18 of index potential values. Additionally, it also produced the highest dextranase activity with 1.5 U/ml at 12<sup>th</sup> hour of incubation. It was therefore selected for further investigation (Table 1).

### 3.2. Morphological Characteristics and Molecular Identification of F4 Isolate

The observation proved that F4 isolate had 0.5 mm to 0.9 mm of colony diameter the shape was

Table 1. Qualitative and quantitative test results of yeast dextranase

| Isolate | Potential index | Maximum activity (U/ml) | Time |
|---------|-----------------|-------------------------|------|
| A4      | 0.87            | 0.2                     | 6    |
| C1      | 1.10            | 0.8                     | 9    |
| F4      | 1.18            | 1.5                     | 12   |

irregular, the edge was filamentous, the elevation was flat, and the color was opaque white. After 24 hours, the culture smelled yeasty or fermented-like odor. Microscopic observation results showed that the size of cells about 1.5 µm to 5 µm. The cells had vacuole and some of them formed budding structure. Amplification results of ITS gene using 5f and 4r primers showed the amplicon size is about 500 bp. Analysis of BLAST showed that F4 isolate had the closest relationship with *Pichia kudriavzevii* strain CBS 2457 with 99% of similarity level. It was supported by phylogenetic tree which proved that both of isolates were on the same clade with the bootstrap value was 67% (Figure 1).

### 3.3. Growth and Enzyme Activity of F4 Isolate

The growth of yeast can be observed through the increase of cell quantity for the incubation time. There were three growth phases which can be analyzed from the growth curve of F4 isolate. They were lagging, exponential and stationary phase. Another phenomenon also can be observed by Figure 2. There were two different peaks which appeared in the enzyme activity curve. A small peak appeared at 5<sup>th</sup> hour when the culture was at a lag phase while the higher one appeared at 12<sup>th</sup> hour when the culture was at initial stationary phase. Therefore, the 12<sup>th</sup> hour of incubation was the best time to produce dextranase with the highest enzyme activity, 1.53 U/ml.

### 3.4. Carbon and Nitrogen Source for Dextranase Production

The effect of carbon sources that was added into the basal medium was investigated. The result indicated that maximal yield of dextranase (0.27 U/ml) was obtained with glucose as carbon source. This value was the highest among all tested carbon sources. The lower yield of dextranase was produced on a medium with fructose and medium with sugar cane juice medium (Figure 3a).

The effect of nitrogen source on the production of dextranase from F4 isolate presented in Figure 3b. It was found that peptone, yeast extract, urea, and

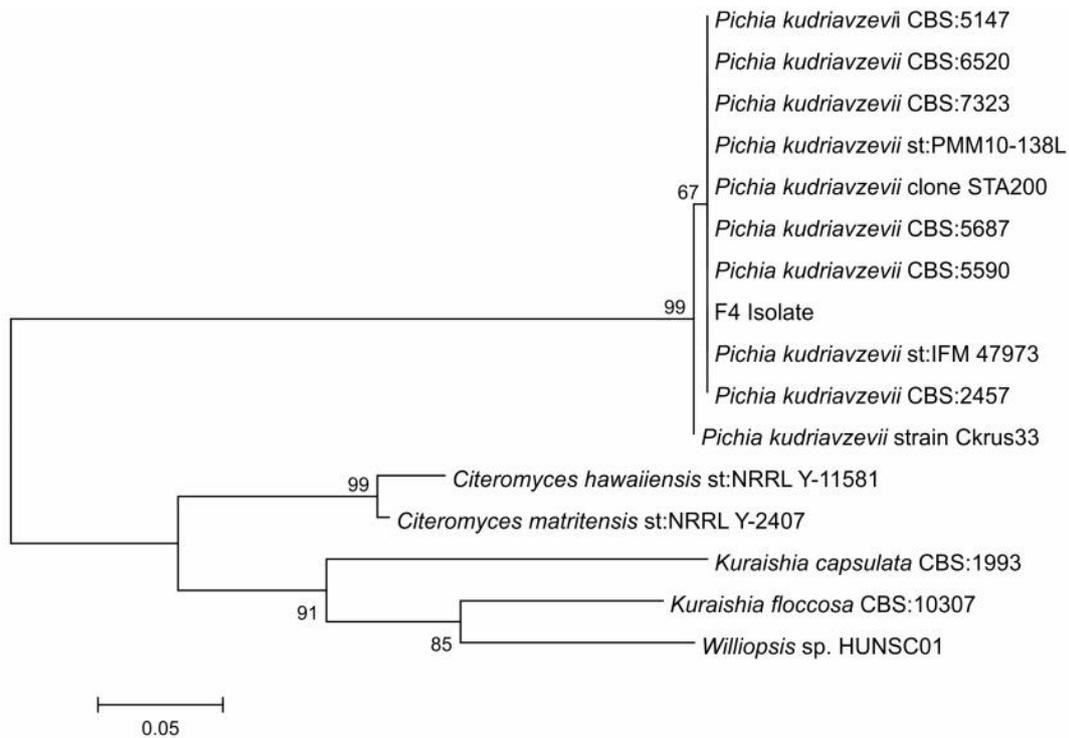


Figure 1. Phylogenetic tree of F4 isolate based on ITS sequences

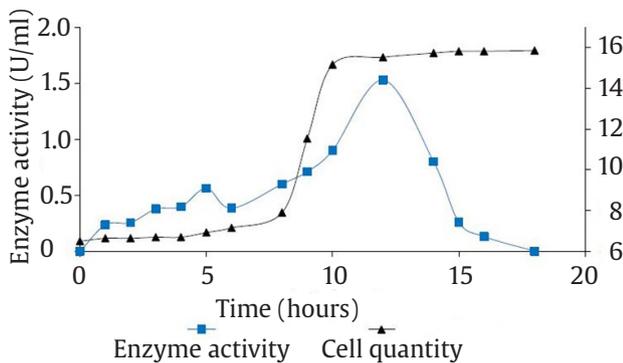


Figure 2. Growth and dextranase activity of F4 isolate on dextran medium for 18 hours at room temperature

ammonium sulphate supported enzyme production as compared to another nitrogen source, tryptone. Moreover, using a combination of yeast extract and peptone as a source of nitrogen produced the highest activity (0.29 U/ml).

### 3.5. Characteristics of Dextranase

Dextranase from F4 isolate had narrow pH range and tend to work at neutral pH. The optimum pH activity was found to be 7 with 0.33 U/ml. Varying the pH from this range adversely affected the enzyme

activity and a loss of 80% enzyme activity was observed at pH 4 (Figure 4a). Quantitative dextranase assays were performed for each temperature. It was found that the greatest dextranase activity occurred at 30°C, and a sharp decrease in activity was observed at 40°C and 50°C (Figure 4b). Investigation of enzyme stability was conducted by storing the enzyme at optimum temperature and 4°C. The result showed that the enzyme activity was more stable at 4°C of storage temperature (Figure 4c).

The cation Na<sup>+</sup> cation played key role as a co-factor by increasing enzyme activity up to 119% for 1 mM of NaCl concentration and 110% for 5 mM of NaCl concentration. Dextranase activity also needed Mg<sup>2+</sup> cation but only at 1 mM of MgCl<sub>2</sub> concentration and more concentration could inhibit the enzyme. The cation K<sup>+</sup> and Ca<sup>2+</sup> were detected as inhibitors of the enzyme by decreasing of dextranase activity in the medium (Figure 4d).

### 3.6. Application of Dextranase to Degrade Dextran

The graphic described that dextranase could degrade dextran on both media. On pure dextran substrate solution, the amount of reducing sugar

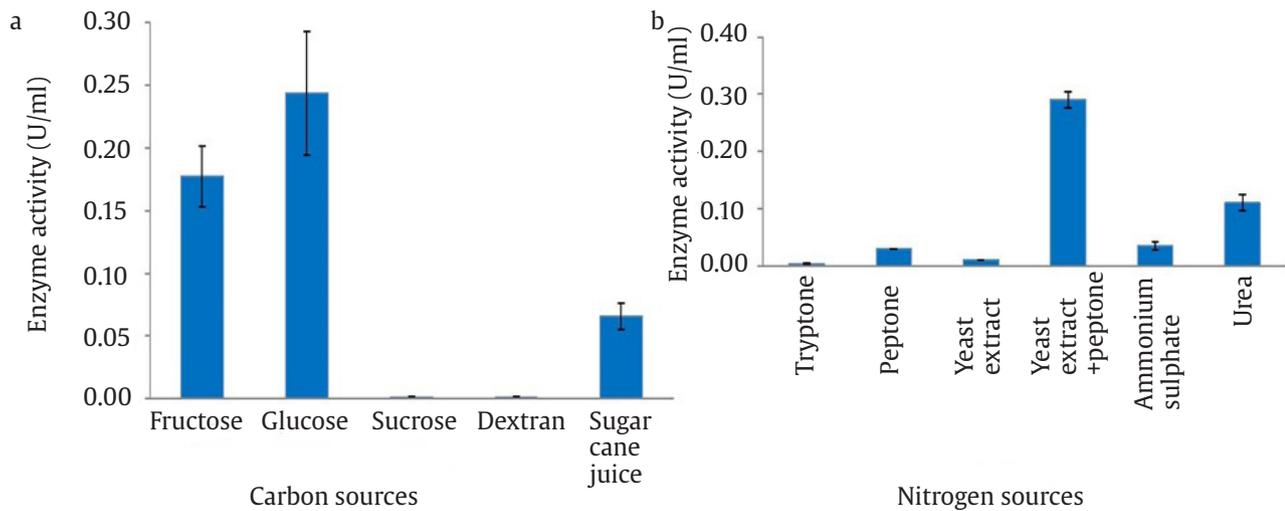


Figure 3. Effect of carbon and nitrogen sources for dextranase production: (a) Carbon sources, (b) Nitrogen sources

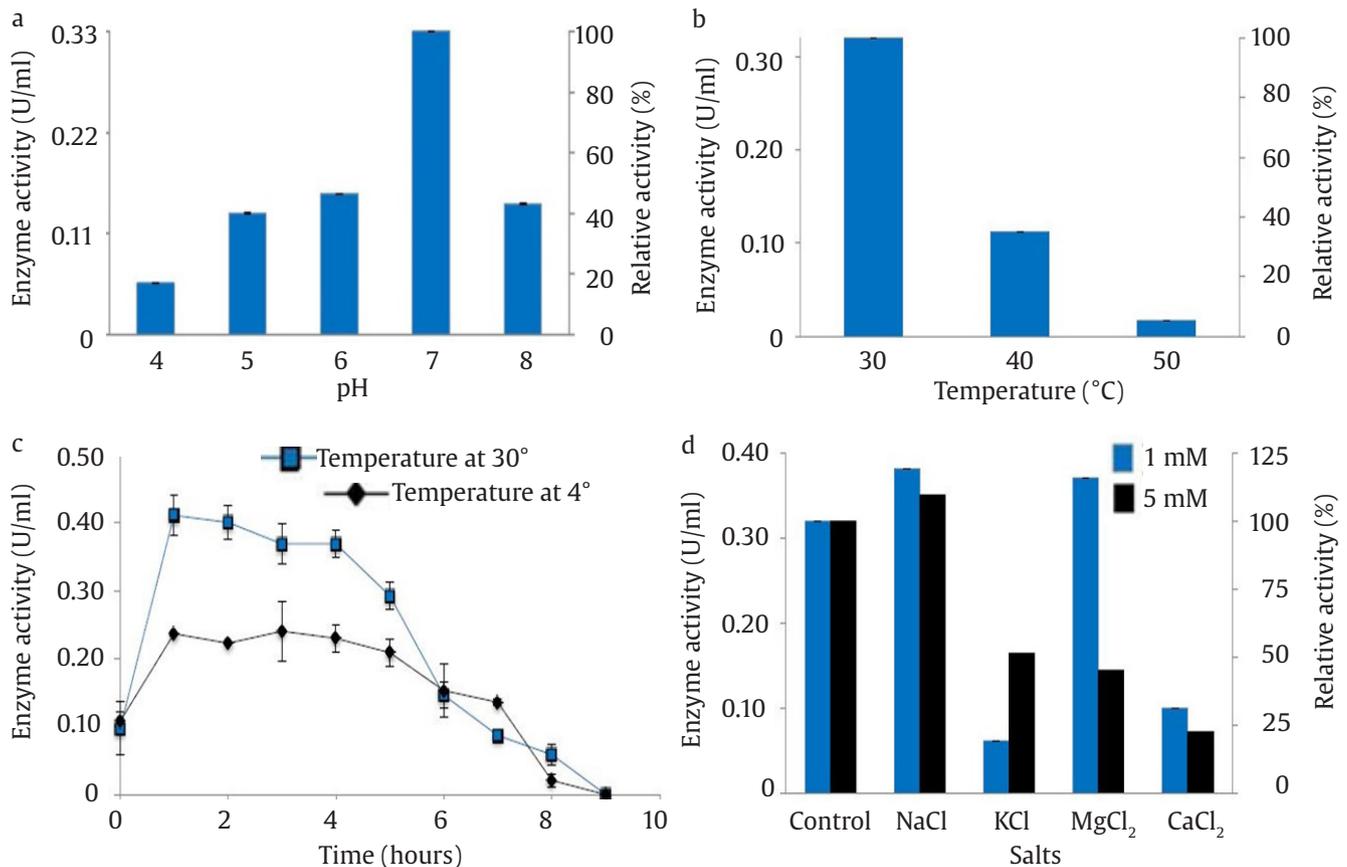


Figure 4. Characteristic of dextranase from F4 isolate: (a) Effect of pH to dextranase activity, (b) Effect of temperatures to dextranase activity, (c) Stability of dextranase at 30°C and 4°C, (d) Effect of cations to dextranase activity

increased rapidly at 1<sup>st</sup> hour and the next hours it levelled off. This condition indicated that dextran was degraded up at the 1<sup>st</sup> hour. It was very different on sugar cane medium. The amount of reducing

sugar was smaller in every hour and increase slowly. Furthermore, the leveling off phase had not performed for 6 hours of incubation time (Figure 5).

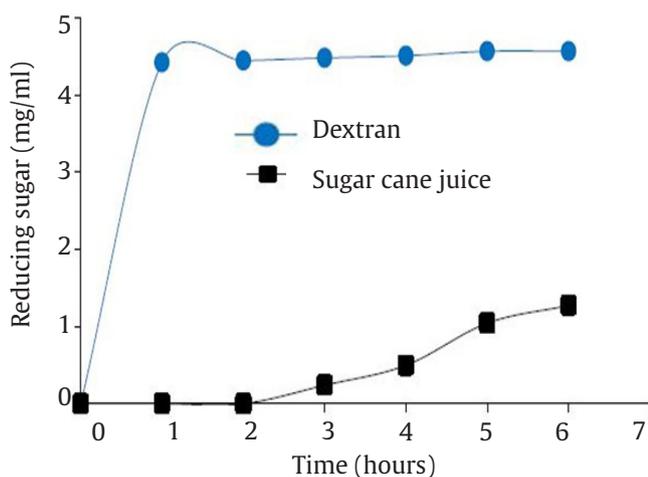


Figure 5. Hydrolysis of dextran on 0.3% dextran and thick sugar juice (54.9 Brix)

#### 4. Discussion

The qualitative test aimed to examine the ability of isolate to produce extracellular enzyme which was proved by formation of hydrolytic zone (Zohra *et al.* 2013). Based on this test, all isolates were able to produce extracellular dextranase but the only F4 isolate that had the highest potential index of dextranolytic. It was also supported by quantitative test which showed that F4 isolate had the largest enzyme activity. But this isolate needed a longer time to produce the highest dextranase activity than the others. It needed 12 hours to achieve 1.5 U/ml of enzyme activity (Table 1). Compared with other dextranase producing yeast such as *Lipomyces starkeyi* that reported by Mahmoud *et al.* (2014), enzyme activity of F4 isolate was still lower. *Lipomyces starkeyi* was able to produce 7.4461 U/ml of enzyme activity.

The use of ITS region for molecular identification due to because of higher variation level than another region in small and large subunit rDNA. The size of amplicon F4 isolate referred to Jerayam *et al.* (2008) who reported that some of yeast which is isolated from hamei had about 440 bp to 880 bp of ITS-5.8S region size. The phylogenetic analysis described the genetic relationship between F4 isolate with *Pichia kudriavzevii* (Figure 1). This genus had been not reported as dextranase producing yeast. At this time, species of *Pichia kudriavzevii* were used to produce phytase for degrading phytate compound on cereal (Hellstorm *et al.* 2012).

Construction of the growth curve and activity curve simultaneously aimed to investigate the growth phase when the maximum of enzyme activity was reached. Figure 2 showed that an increase in dextranase activity indicated that dextranase was inducible enzyme. According to Khalikova *et al.* (2005) synthesis of dextranase was induced by the existence of dextran in pure form or residual form on a medium. The dextranase activity curve of F4 isolate also showed two peaks. It indicated that dextranase was an enzyme system consisted of endoenzyme and exoenzyme (Khalikova *et al.* 2005). The small peak appeared at lag phase at the 5<sup>th</sup> hour of incubation time. At this phase the present of simple sugars was a result of endoenzyme activity that broke down dextran randomly. The increase of cell biomass that was followed by the increase of enzyme activity indicated that dextran was used as a carbon source to support the yeast growth (Figure 2).

The maximum activity was reached at initial stationary phase. These results were in agreement with previously reported results of dextranase from *Penicillium aculeatum* NRRL-898 with 101250 U/ml of enzyme activity (Mahmoud *et al.* 2014). Baktir *et al.* (2005) also reported that dextranase from thermophiles microbe had maximum activity ( $13.97 \times 10^{-3}$  U) at the initial of stationary phase and declined at stationary phase. Mahmoud *et al.* (2014) stated that reaching maximum cell mass will increase accumulation of dextranase. The maximum activity of dextranase related to cell growth that need much carbon sources. The second peak of enzyme activity may be caused by activity of exoenzyme which break down branching linkage of dextran into smaller sugar (Khalikova *et al.* 2005). Decrease of enzyme activity in stationary phase might be caused low substrate level, change of the enzyme active site, and feedback inhibition (Figure 2).

Microbes had preference to select a carbon source that was needed for their growth. In addition of carbon source besides of dextran into medium aimed to stimulate their growth before dextran was used. Glucose was reported could produce the highest dextranase activity (Figure 3a). Glucose is simple monosaccharide that can be uptake directly into a cell without extracellular hydrolysis process. According to Weinhandl (2014) glucose was favorable carbon for microbial growth. The existence of glucose can increase the microbial growth rate and

repressed enzyme synthesise like dextranase. But, when glucose was used up, microbes will produce dextranase to degrade dextran as carbon source.

Maximum cell biomass can raise dextranase production (Gorke and Stulke 2008). Beside of glucose, fructose could increase dextranase production too, because it was also monosaccharide which could be used easily by yeast to initiate the growth. The activity of dextranase also existed on sugar cane juice medium. Sugar cane juice provides some of carbon sources and nitrogen source for microbial growth (Thai and Doherty 2011). Meanwhile the activity of dextranase was induced by the existence of dextran on sugar cane juice. On medium contained dextran as sole carbon source produced very low dextranase. These indicated that dextran was not suitable carbon source to initiate yeast growth, thus the dextranase production was also low (Figure 3a).

Various organic and inorganic nitrogen sources played a key role in dextranase production. Nitrogen was used by yeast to build the component of cell and to form macromolecules like protein. Each species of yeast had different nitrogen need. The selection of nitrogen source showed that combination of yeast extract and peptone could produce dextranase with the highest activity (Figure 3b). Similar to previous studies conducted by Zohra *et al.* (2013) that addition of yeast extract and peptone into medium simultaneously can increase dextranase production to 61.3%. Qader *et al.* (2006) also reported that addition of single nitrogen is insufficient to higher dextranase yield.

The enzyme activity was influenced by several factors like pH, temperature, and metal compound that can be an activator and an inhibitor of the enzyme. pH gave effect on the active site of the dextranase. At optimum pH, dextranase would produce higher activity to degrade substrate, but at unappropriate pH, the enzyme could get denaturation. The optimum pH of F4 isolate was 7 (Figure 4a). Dextranase from *Chaetomium gracile* reported by Hattori *et al.* (2014) had optimum pH at around 5.5 and was stable in a pH range from 5.5 to 11.0. According to Purushe *et al.* (2012) the pH optimum of enzyme related to the origin habitat of the isolate. The optimum pH of the enzyme was similar to with a pH of sugar cane juice after clarification and evaporation process (Figure 4a).

Temperature also influenced dextranase activity. Selection of temperatures (30°C, 40°C, and 50°C)

were based on the temperature along the sugar manufacturing process. According to Eggleston and Monge (2005) the sugar manufacturing processes operated at temperature ranging from 50°C to 80°C. The juice is clarified with dextranase at around 30°C and the mixed juice or syrup is treated at 60°C. The optimum temperature of this enzyme was 30°C (Figure 4b). It indicated that dextranase could work well along juice clarification process. That enzyme was categorized into mesophilic enzyme. Meanwhile dextranase from fungi *Penicillium aculeatum* NRRL-896 showed optimum activity in the range of 35-45°C (Mahmoud *et al.* 2014).

The storage of enzyme at 4°C made the enzyme more stable than the storage at optimum temperature. It was caused a low temperature could keep the protein structure of the enzyme. Low temperature also could decrease the enzyme activity due to protein coagulation. Different enzyme stability in two storage conditions was caused by an enzyme which was stored at 4°C had to be adapted at an incubation temperature of enzyme assay (Figure 4c). According to Suhartono (1989), enzyme stability depended on the hydrophobic bridge on its molecule while the decrease of enzyme activity is caused by enzyme denaturation. Sugar cane juice contained mono and divalent cations such as K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> that played role as co-factor or even inhibitor for dextranase (Thai and Doherty 2011). The cation Na<sup>+</sup> played role as a co-factor for the dextranase. It was in agreement with Baktir *et al.* (2005) who reported that the addition of 50 mM NaCl could raise dextranase activity and more than 50 Mm would decrease the activity. Meanwhile the cation Ca<sup>2+</sup> and K<sup>+</sup> played role as inhibitor for dextranase (Figure 4d). Previously also reported by Wang *et al.* (2014) using dextranase from *Arthrobacter oxydans* KQ11.

To prove that dextranase from F4 isolate can be applied to eliminate dextran on sugar cane juice, measuring product like glucose from substrate hydrolysis by the enzyme was needed to be done. The increase of product indicated the capability of enzyme to degrade dextran at real condition. Dextran-containing juice was used as the main medium to ensure that dextranase was really able to degrade dextran in the sugar manufacturing process. Therefore sugar cane juice was made exactly similar with sugar cane juice in the sugar industry. The pure dextran solution was used to compare the work of enzyme between two different conditions.

The result proved that dextranase from F4 isolate could hydrolyze dextran both in pure and in mixed dextran substrate with different hydrolysis rate. In pure dextran substrate, the existence of other component besides dextran was very low (Figure 5). In contrast, sugar cane juice contained a lot of other components besides dextran such as glucose, galactose, sucrose, raffinose, etc. Shamsolahlar *et al.* (2002) stated that glucose, galactose, sucrose, and raffinosa could be a competitive inhibitor of the enzyme. If the enzyme had linked to the inhibitor, the reducing sugar could not be produced (Sun *et al.* 1995). The dextran hydrolytic process needed water to be broken up into H<sup>+</sup> ion that will bind the product and OH<sup>-</sup> ion that will bind another product. Long incubation period causes water loss and may cause a decrease of dielectric constant to separate different ion. This will facilitate the metallic ion to bind the enzyme (Suhartono 1989).

In laboratory studies at pH 5.0°C and 50°C with 1500 ppm initial concentrations of dextran in the presence of 15% sucrose showed that the dose of 16 ppm of Herbertec-Dextranase hydrolyzed 90% of the polysaccharide in 10 minutes while the higher concentration of dextran had a lower level of hydrolysis. The observation also proved that the reduction of the reaction temperature to 35°C caused a decrease of the hydrolysis level reaching 90% for an initial concentration of dextran of 500 ppm. In industrial scale performed in juice at a dose of 16 ppm of Herbertec-Dextranase provided a hydrolysis of 85% of the 1600 ppm of dextran present in juice with a reaction time of 10 minutes (Jimenez 2009).

In conclusion, this research has provided information about potential yeast producing dextranase from Indonesia, production and characterization of its dextranase to be applied in the sugar industry to reduce dextran on sugar cane juice. The selection results stated that F4 isolate was selected with the maximum enzyme activity (1.53 U/ml) which is achieved at the initial stationary phase at 12<sup>th</sup> hours of incubation time. The isolate also showed particular properties of yeast by the presence of vacuole inside the cells and budding cells. Molecular identification showed that F4 isolate was closely related to *Pichia kudriavzevii* with 99% of similarity value. The highest production of dextranase was induced by the supplementation of glucose and combination of yeast extract and peptone. The enzyme had optimum working condition at

pH 7 and temperature at 30°C. The stability of enzyme showed that the enzyme activity was more stable at 4°C of storage temperature. The cation Na<sup>+</sup> played key role as a co-factor while K<sup>+</sup> and Ca<sup>2+</sup> were detected as inhibitors of the enzyme. Dextranase from F4 isolate can hydrolyze dextran both in pure and in the mixed dextran substrate, but with a lower hydrolysis rate.

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