Frontiers in Scientific Research and Technology

Online ISSN: 2682-297
Print ISSN: 2682-2962
http://fsrt.journals.ekb.eg

FSRT J 1 (2020) 74-80 DOI: 10.21608/fsrt.2020.31958.1016

Glycerol production by the UV-mutant marine yeast *Wickerhamomyces anomalus* HH16-MU15 via simultaneous saccharification and fermentation of fruit peels

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ARTICLE INFO

Article history:
Received 6 June 2020
Received in revised form 27 June 2020
Accepted 28 June 2020
Available online 29 June 2020

Keywords
Glycerol;
fruit peels;
hydrothermal treatment;
fermentation;
Wickerhamomyces anomalus.

ABSTRACT

Agro-industrial wastes in Egypt are indefinite and sustainable substrate for production of numerous industrially significant products. Using of these wastes as raw materials can reduce both the production cost and the environmental pollution. The present research aims to produce glycerol from fruit peels by the UV-mutant marine yeast *Wickerhamomyces anomalus* HH16-MU15. Fruits (banana, orange, mango and pomegranate) peels were pretreated by mechanical and hydrothermal methods before their fermentation into glycerol. The maximum total reducing sugars (5.792 ± 0.16gl^{-1}) was obtained from mango peels followed by orange peels (5.33 ± 0.21gl^{-1}) after their hydrothermally pretreatment. After fermentation, the maximum cellulase activity (8.911 Uml^{-1}), total reducing sugars (48.59 gl^{-1}) and glycerol yield (35.25 gl^{-1}) by *W. anomalus* HH16-MU15 were achieved in the hydrolysate of hydrothermally pretreated orange peels at 96 hrs. The current investigation suggests the using of orange peels for production of glycerol and other valuable products by *W. anomalus* HH16-MU15.

1. Introduction

Polyols (polyhydric alcohols) are sugar derivatives in which the aldo or keto group has been reduced to the corresponding hydroxyl group with the general formula $HOCH_2$ $(CHOH)_nCH_2OH$. They are one of the most abundant classes of organic molecules that have many biologically significant roles in marine yeasts [1]. Marine yeasts produce large amounts of polyols during their growth in their natural habitats to overcome the high external osmotic pressure of the marine environment [2]. It has been suggested that polyols may mimic the structure of water and maintain an artificial sphere of hydration around macromolecules preventing peroxidation of lipids that cause cell damage [3]. The most predominant polyol as an osmolyte in marine yeasts is the glycerol [4]. Glycerol (1,2,3-propanetriol) is used in manufacturing of food additives, cosmetics, paints, paper and leather as well as in the production of pharmaceutical formulating agents [5]. Moreover, it is a significant precursor for production of appreciated chemicals such as biodiesel, butanol, hydrogen, acrolein, citric acid, dichloro-2- propanol (DCP), 1,3-propanediol (1,3-PD), and 1,3-dihydroxyacetone (DHA) [1].

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Conversion of agro-industrial wastes such as fruit peels to glycerol can be an economic promising task. Furthermore, the use of seawater based medium for glycerol production could be a promising strategy for saving fresh water [6]. Fruit peels pose threat to the Egyptian environment by increasing the contamination and represent a loss of valuable biomass and nutrients. In the past years, the Egyptian fruit peels have been disposed in solid municipal wastes which cause environmental pollution and formation of unpleasant odors [7]. Currently, this is not economic since the presence of high amounts of nutrients (sugars, proteins, and fats) in these fruit residues [8]. Therefore, the treatment of these wastes and using them for production of glycerol instead of their disposal represents a sustainable eco-friendly alternative way. The fermentation of these wastes directly is very difficult and unsustainable because they have a complex structure of the three main components; cellulose, hemicellulose, and lignin with variable proportions [9]. They should be subjected to a pretreatment process in order to separate the cellulose-lignin complex proper to their fermentation by microorganisms [10].

Various mechanical, physical, chemical and biological processes can be used for agro-industrial wastes pretreatment [11-13]. Mechanical pretreatment is grinding step that usually performed to reduce the particle size crystallinity before subsequent processing steps. Physical pretreatment includes the hydrothermal pretreatment, also

known as hot-compressed water treatment or auto-hydrolysis treatment, is an environmentally friendly technology at which biomass is treated with water at high temperature and pressure [14]. The second step in the bioconversion of fruit peels is the enzymatic hydrolysis of the physically or chemically pretreated waste. The biological conversion of lignocellulosic biomass to valuable products is mainly depend on the efficiency of enzymatic degradation of cellulose to reducing sugars [15]. Marine yeasts have gained considerable attention as a source of hydrolysis enzymes such as cellulase [16]. Therefore, simultaneous saccharification and fermentation (SSF) of fruit peels to glycerol by a marine yeast in single step will reduce the time and the overall cost of the process [17].

The current work was aimed to explore the capability of the mutant marine yeast *Wickerhamomyces anomalus* HH16-HH5 to utilize fruit peel hydrolysates as sustainable substrates for glycerol production. Moreover, this study has been evaluated the efficient pretreatment method (hydrothermal) for hydrolysis of fruit peels to release fermentable sugars and examined subsequent simultaneous saccharification and fermentation process.

2. Materials and methods

Microorganism source:

The yeast strain *Wickerhamomyces anomalus* HH16-MU5 used in the present study, is UV-mutant of the wild yeast *W. anomalus* HH16 which have been isolated from marine sediment, UV-treated and tested for glycerol production previously [5].

Preparation of yeast inocula:

A loopfull of 48 hrs old yeast culture was inoculated into 50 ml sterilized yeast extract-malt extract (YM) broth medium and then incubated for 48 hrs on a rotary shaker (150 rpm) at 30 ^{o}C. The YM broth medium consists of (g/l seawater): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0 and dextrose, 10.0 [13]. The medium pH was adjusted to 5.5 ± 0.2 using 0.1 N HCl and 0.1 N NaOH.

Fruit peels source:

Different four types of fruit peels banana (*Musa acuminate)*, orange (*Citrus sinensis*), mango (*Mangifera indica*) and pomegranate (*Punica granatum*) peels were collected from different natural juices shops located in Suez city, Egypt.

Fruit peel hydrolysates preparation:

Mechanical pretreatment

Fifty grams of each fruit peel (banana, orange, mango peels and pomegranate peel) was initially washed individually with tap water, air-dried for two hours and dried in a convection oven at 65 ºC for 24 hrs [10]. The dried wastes were then milled using an electric mill to obtain particle size of approximately 1 mm in order to reduce the cellulose crystallinity to enhance the saccharification process (Figure 1).

Fig 1: Shapes of grinded dry fruit peels after the mechanical pretreatment; a) banana peels, b) orange peels, c) mango peels and) pomegranate peels.

Hydrothermal pretreatment:

Fifty grams of each milled dry fruit peel was soaked in distilled water at a liquid to solid ratio of 1:20 (w/v) in 2 liters Erlenmeyer flask. The flasks were then autoclaved at 121 ^{o}C for 60 minutes. The residue was filtrated using cheese cloth and then dried in an oven at 65 ^{o}C for one day [18].

Simultaneous saccharification and fermentation (SSF) process:

Simultaneous saccharification and fermentation (SSF) combines the enzymatic hydrolysis step and the fermentation step in order to make the conversion process more efficient. The used medium is composed of (gl^{-1} seawater): yeast extract, 1; peptone, 1; KH_2PO_4, 1; $MgSO_4$, 0.5 and was supplemented with 50 g treated fruit peel. The medium pH was adjusted to 6.0 ± 0.2 using 0.1 N HCl and 0.1 N NaOH.

The simultaneous saccharification and fermentation (SSF) process was conducted in 250 ml Erlenmeyer flasks separately, each containing 100 ml medium inoculated with 20% of the selected mutant yeast inocula (10^6 cells /ml). The flasks were incubated at on shaker incubator (150 rpm) at 30 ^{o}C and testing samples were taken at different fermentation periods (24, 48, 72, 96, 120, 144 and 168 hrs). In each hydrolysate, the total reducing sugars, cellulase activity and glycerol were assayed as follows.

Total reducing sugars (TRS) determination:

The total reducing sugar liberated during the enzymatic assays was quantified by the dinitrosalicylic acid (DNS) method [19] using glucose as a standard. DNS reagent was prepared by dissolving 10.0 g of 3,5-dinitrosalicylic

acid, 200 g of sodium potassium tartarate, 10.0 g of sodium hydroxide, 2.0 g of phenol and 0.5 g of sodium sulfite in one-liter distilled water. Three ml of diluted fermentation medium was added to 3 ml of DNS reagent and the mixture was boiled for 5 minutes. The mixture was cooled down to room temperature, 1ml of Rochell salt (40% potassium sodium tartarate) was then added and absorbance was measured at 510_{nm} by UV-9200 VIS spectrophotometer.

Carboxymethyl cellulase (endo-β-1,4 glucanase) activity assay:

Carboxymethyl cellulase (endo-β-1,4 glucanase) activity was assayed by adding 0.5 ml of the diluted hydrolysate to 0.5 ml of 1% carboxymethyl cellulose solution and incubated for 30 minutes at 50 ^{o}C**.** After incubation, 3 ml of DNS reagent was added and boiled for 5 minutes. The absorbance was measured at 540 nm against the spectro zero by UV-9200 VIS spectrophotometer. The carboxymethyl cellulase (CMCase) enzymatic activity was assayed according to [20] using the following equation:

$$\text{CMCase} = \frac{0.185}{\text{Enzyme concentration to release 0.5 mg glucose}} \text{ units ml}^{-1}$$

All enzymatic assays were carried out in triplicate and the mean values were calculated and reported. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 µmol of the corresponding product (glucose equivalent) per minute, under the assay condition used.

Glycerol determination:

Glycerol was quantitatively measured using sodium periodate method according to [21]. For each experiment, total glycerol concentration (*P*) and volumetric glycerol productivity (Q_p) were calculated according to the following equation:

$$Q_p = dP/dt$$

Whereas volumetric glycerol productivity (Q_p) was calculated as grams of glycerol formed per liter per hour ($gl^{-1}h^{-1}$).

3. Result and Discussion

3.1 Evaluation of the hydrothermal pretreatment process on fruit peels hydrolysis before the fermentation process:

Data presented in Figure 2 reveal that the hydrothermal pretreatment of grinded dry fruit peels can effectively afford the recovery of the reducing sugars from these wastes. [22] reported that the water-steam (hydrothermal) pretreatment is suitable efficient method for recovering the reducing sugars from fruit wastes. At hydrothermal pretreatment, pressure is gradually released, and the steam expands within the lignocellulosic complex, causing them to be separated, disrupted and simple sugars can be released in the hydrolysate [23]. In this study, the obtained maximum reducing sugars (5.792 ± 0.16gl^{-1}) was resulted from hydrothermally pretreated mango peels, followed by orange peels with total reducing sugars 5.33 ± 0.21gl^{-1}.This result is consistent with [24], who investigated that among the four fruit wastes (banana, papaya, pineapple and mango peels), mango peels contain larger amounts of reducing sugars up to 40% (w/v). This is might be attributed to the softens and low lignin content in the outside peels of mango fruits.

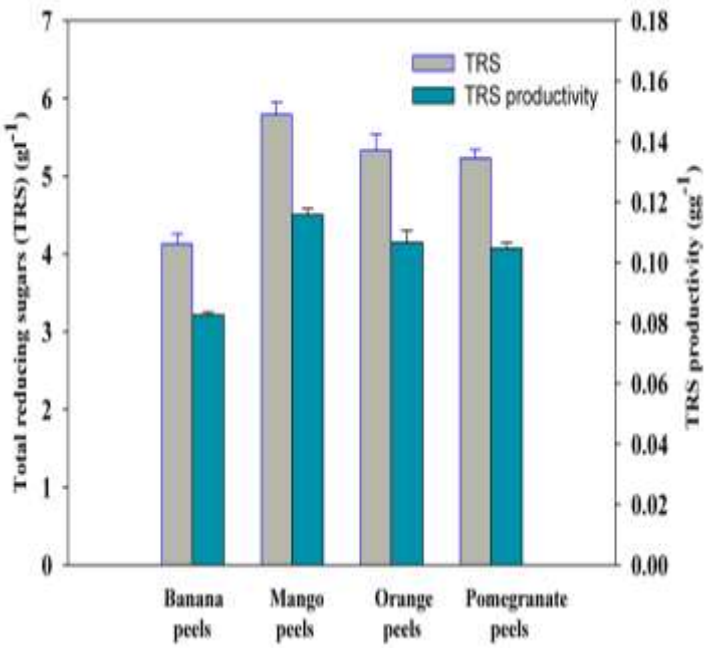

Fig 2: Total reducing sugars (TRS) and sugars productivity in the hydrolysates of the hydrothermally treated fruit peels before fermentation process.

3.2 Effect of the simultaneous saccharification and fermentation process on the total reducing sugars and carboxymethyl cellulase activity:

Data in Figure 3 reveal that simultaneous saccharification and fermentation of the different fruit peels using the mutant isolate *W. anomalus* HH16-MU5 resulted in suitable yields of total reducing sugars. However, statistical analysis of the results indicated that different fruit peels might exhibit various effects on the cellulase activity of the yeast and subsequently the liberated total reducing sugar. The maximum CMCase activity 8.911 $Uml^{-1}$and the highest total reducing sugar 48.59 gl^{-1} were recorded in the orange peels hydrolysate after 96 hrs of fermentation. On the other hand, the minimum CMCase activity 2.335 Uml^{-1} was recorded after 168 hrs using the banana treated hydrolysate with the lowest reducing sugar recovery 13.159 gl^{-1}. The reason could be that the hydrolysis of various lignocellulosic materials release different mixture of hexose sugars (e.g. glucose, mannose), pentoses (e.g. xylose, arabinose) and inhibitors [25].

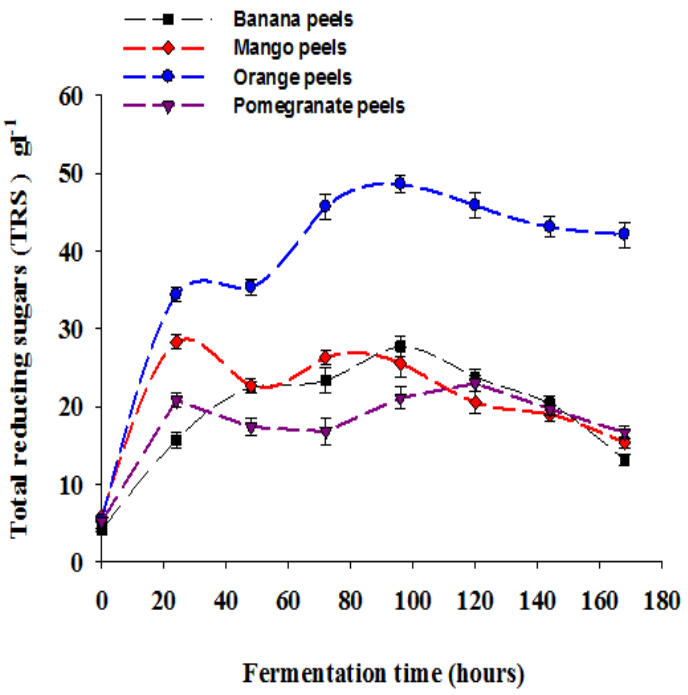

Fig. 3: Total reducing sugars production during saccharification at different fermentation times using the mutant isolate *W. anomalus* HH16-MU5.

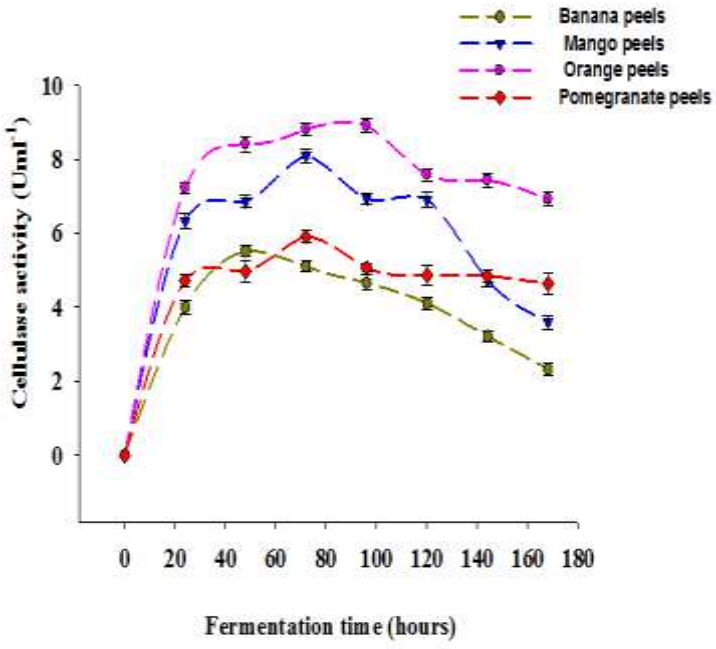

Fig. 4: Carboxymethyl cellulase activity of the mutant isolate *W. anomalus* HH16-MU5 on the pretreated fruit peels at different fermentation times.

According to [26], steam explosion and liquid hot water pretreatments of fruit peels can generate soluble inhibitors which hamper the cellulase enzymatic activity and hence the released reducing sugars. Toxic and inhibitory compounds vary with the pretreatment method and the waste type that might include soluble sugars, organic acids (levulinic, acetic and formic acid), and furan derivatives (hydroxymethyl fulfural) [9,27].

The obtained results in Figure 4 indicated that the mutant isolate *W. anomalus* HH16-MU5 has an efficient cellulase production system, therefore it could be a candidate for the enzymatic hydrolysis of lignocellulosic wastes that might reduce the time and the overall process cost. [17] reported that the simultaneous saccharification and fermentation using single yeast isolate can be considered as a cost-prohibitive due to the high cost of commercial cellulase enzymes. According to [28], cellulases isolated from marine fungi, especially yeast, have shown their potential for bioconversion processes which can have role in bioenergy-based industries. Several marine yeast genera such as *Aureobasidium*, *Candida, Rhodotorula* and *Wickerhamomyces* could be a source of cellulases and other valuable hydrolytic enzymes [5,29-30].

3.3 Glycerol production from fruit peels hydrolysates by W. anomalus *HH16-MU5:*

Results indicated that the simultaneous saccharification and fermentation of fruit peels to glycerol by the mutant marine yeast *W. anomalus* HH16-MU15 is sustainable and can be feasible. Moreover, the use of seawater-based medium can provide a huge impact on freshwater crisis especially in coastal areas where freshwater is priceless. There is a clear variation in the glycerol production by the mutant isolate *W. anomalus* HH16 MU5 at the different fruit peels hydrolysates (Figure 5). The maximum glycerol yield 35.25 gl^{-1} was achieved with orange peels hydrolysate at 96 hrs incubation at 30°C **(**Figure 5C). This indicates the possibility of using orange peels as a promising substrate for glycerol production by yeast fermentation. In Egyptian citrus industries, the traditional re-use of orange peel and pulp wastes could be impractical and has a high cost, therefore peel and pulp wastes are accumulated in the soil by these industries that might cause serious environmental problems [31]. Orange peels could have the potential for valuable products as they are low cost with high renewable production rate [32].

The mango peels hydrolysate was the second best substrate for glycerol production with volumetric glycerol productivity 0.838 $gl^{-1}h^{-1}$ and glycerol production 31.67 gl^{-1} after a fermentation times 24 and 96 hrs (Figure 5B).These results were in accordance to [24], who revealed that mango peels can be one of the potential and novel raw materials for yeast fermentation.

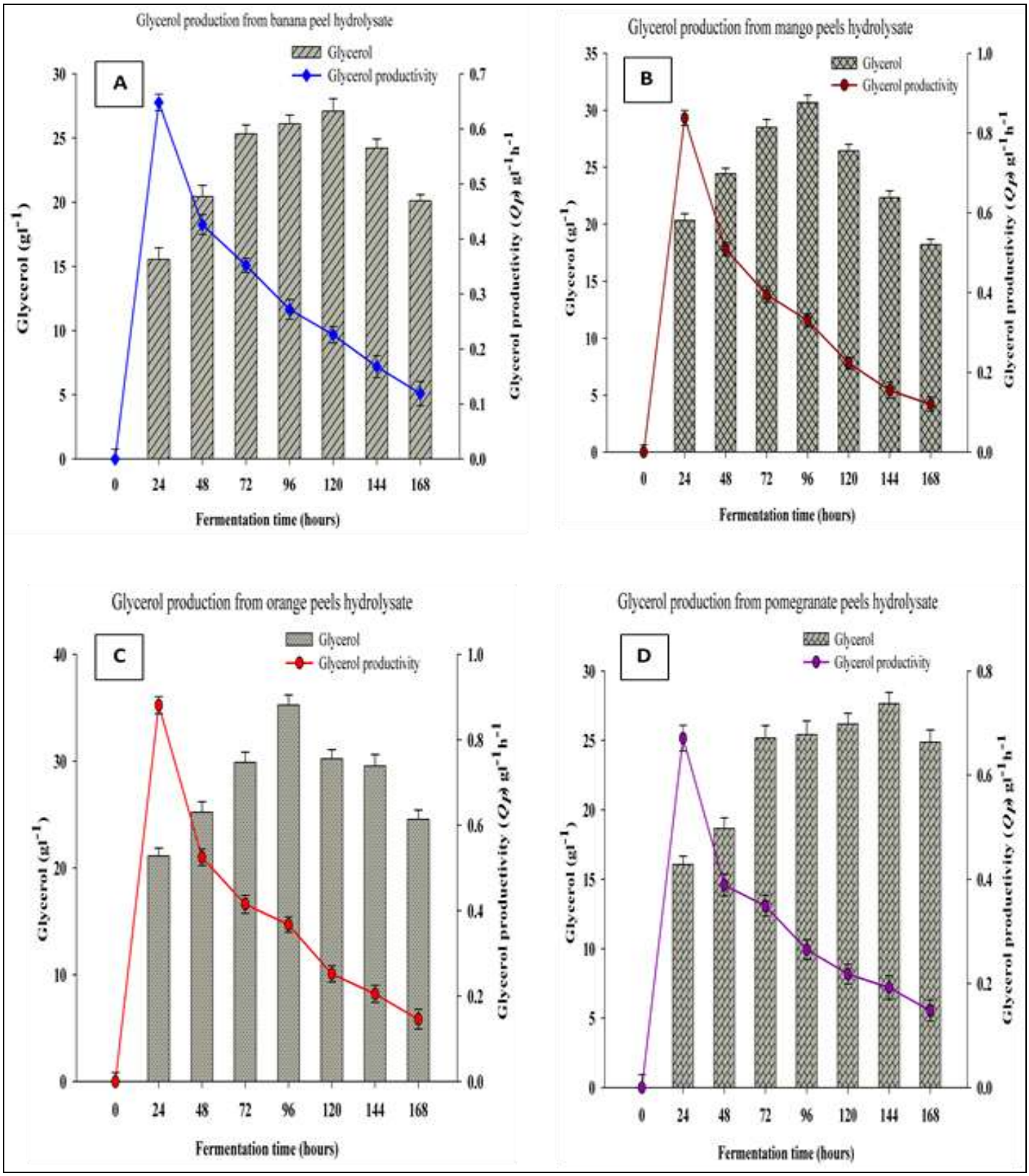

Fig. 5: Glycerol yields and glycerol productivity of the hydrothermally pretreated fruit peels at different fermentation times by the mutant isolate *W. anomalus* HH16-MU5.

Although the banana peels hydrolysate showed a good sugar recovery (Figure 3), the recorded glycerol production rate was weak resulting in 27.11 gl^{-1} glycerol after 120 hrs (Figure 5A).This might be due to the phenolic acids, tannins and lignin that found in banana plant cell walls; the presence of phenolic and carboxyl groups can inhibit or decrease the fermentation efficiency [33].

4. Conclusion

The current investigation reveals that the UV-mutant marine yeast *W. anomalus* HH16-MU15 could be a candidate isolate for simultaneous saccharification and fermentation of the fruit peels to glycerol. Furthermore, the use of seawater based medium for glycerol production could be a promising strategy for saving fresh water. The hydrothermally pretreated orange peels can be good substrate for glycerol and cellulase production. The present study confirms that the efficiency of enzymatic hydrolysis and fermentation process is mainly depending on the pretreatment process and the type of agro-industrial waste.

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