



Impact of Microbial Synergism on Second Generation Production of Bioethanol from Fruit Peels Wastes

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Abstract

Fruit peels annually accumulate in huge amounts, fruit wastes are rich in lignocellulosic component which can be recovered into mono simple carbohydrates able to utilize for bioethanol production. The enzymatic hydrolysis of lignocelluloses is known to be a key to the second-generation biofuel, the challenge is the still expensive enzymes involved in the saccharification process, loss of the most hemicellulose pentose sugars which were non-fermentable as base, beside the presence of hampers lignin thus require to resolve its problem. As part of study, followed the cost-effective means for bioethanol producing from four fruit peels (Banana, Orange, Mango and Watermelon), yielding of enzymes from co-fungal cultivation carried by *Aspergillus niger* and *Phanerochaete chrysosporium*, followed by co-fermentation of the saccharified sugars using yeast belong to *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* which contributed for bioethanol production in 8 L lab-scale reactor. The fermented sugars recovering was reached to 27.77 g.l⁻¹ from banana peels which were found to be good exploited as potential raw source, co-fungal enzymatic hydrolysis followed by yeast co-fermentation led to substantial yield by 10.74 g.l⁻¹, the adding of

calcium oxide increased the purity which leads finally to 97.5 wt % of pure bioethanol. Thrust towards fossil fuels replacement with renewable clean fuels such as bioethanol by using fruit peels residuals which are considered renewable energy source may also help in CO₂ mitigation.

Keywords: Fruit peels, Bioethanol, Fungi co-cultivation, Yeast co-fermentation

1 Introduction

The huge amounts of consumed fresh fruit encouraged the expansion use of its peels residues to improve their economic productivity competitiveness (Cutzu and Bardi 2017). Most of fruits after its consumption generates about 50% of their weight as un-eatable waste, its utilization to generate bioethanol from their huge effluents as substrates will indeed reduce the processing costs and environmental load for agro-wastes management (Hu et al 2011). While the most of global food waste are arises from fruits and vegetables residues, fruits peels are alone account about 37% of the total agricultural waste according to the statics of Food and Agriculture Organisation (Makkar 2013). Also, the major organic matter generated daily by farms, household's consumption and food

processing manufacturers are fruits rinds and peels, these disposed organic materials that pose an environmental risk due to their high biological oxygen demand (BOD) (Ververis et al 2007). These fruit residues are of no commercial importance and mostly disposed of in municipal dumps by large quantities, which causes of serious organic pollution (Oberoi et al 2013). Among them, the major contents of fruits peels are cellulose and hemicellulose with significant amounts of lignin placed between them, these lignocellulosic are considered a great renewable raw material for competitive production of bioethanol in the open market (Makkar 2013). Unfortunately, peels cells are mainly composed from complex fibers, these fibers are covalently hemicelluloses coupled with lignin and linked by hydrogen bonds with cellulose (Chen and Fu 2016). The complex compound ties all the constituents of peels which is the principal barrier to their uses valorisation, therefore a process of pre-treatment is inevitable to separate hemicellulose and lignin from cellulose (Demirbas 2011). Many approaches have been developed to treat hemicelluloses and lignocelluloses biomass to separate their chemical components, in which bioethanol production is the major target (Lee et al 2014). Thus, the primarily chemical components that can be fermented to alcoholic component are simple sugars, the latter cannot get it without other treatment step named saccharification process, there is no substitute of the latter process for separating them from polysaccharides to make fermentation possible (Chen and Fu 2016). Due to lignin properties, it is become the main obstacle which influences the digestibility of the biomass. Therefore, the high resistant lignin crystalline structure could be lowering the biomass digestibility. Whereas the stimulation of lignin degradation would be stimulating its digestibility (Agbor et al 2011). Overall, the commercial production has not yet been implemented because of production cost elevation based on available pre-treatment technologies (Dien et al 2003). Even though dilute acid and hydrothermal pre-treatment are the most common and economic than the other pre-treatment

methods, but some of these methods would be more harmful to the environment than others as the chemical free pre-treatment such as hydrolysing enzymes which is environmentally attractive solutions although it is still high cost (Rocha-Meneses et al 2019). Microbial enzymatic hydrolysis is more promising than any others chemical or physical hydrolysis since lower utility costs with highest productivity (Hu et al 2011). The commercially available enzymes mixtures are produced by *Aspergillus* species especially *A. niger* strains are the most commonly industrial fungus which produced a broad range of degrading exo-enzymes, such fungal enzymes are essential to convert complicated plant polymers into simple molecules that can be up-taken into their cells (Arya et al 2001). Also, the enzymatic treatment would also avoid initially physical or chemical treatments for the easily sugars recover without various inhibitions (Hu et al 2011). Many authors pointed that, during fungal natural life cycle most fungi live in mixed communities within symbiosis or competition particular biotope and may be colonized mainly by several *Aspergilli* species (Arya et al 2001, Hu et al 2011). Thus, the fungi co-cultivation may result more in more efficient enzyme mixtures for various applications than those result from monoculture (Arya et al 2001). The fungal co-cultivations were previously described for efficient productivity of degrading enzymes, the ascomycete strains of *A. niger* and the basidiomycete white rot fungus *Phanerochaete chrysosporium* could improve the activity of cellulolytic and hemi-cellulosic enzymes and improve ligninolytic enzymes production (Verma and Madamwar 2002). Another side, the yeasts belonging to *Saccharomyces* genus is commonly used for alcoholic fermentation, especially the typical species of *S. cerevisiae* is ordinary considered the common fermentation species, it has the highest yield and ethanol tolerance (López-Malo et al 2013). In addition, the most of current theoretical for bioethanol production were by *S. cerevisiae* which aptitude to grow at low pH and elevated temperature, also its capable to grow in presence of inhibitors, the using of converted sugars by *S.*

cerevisiae was far better from enzyme hydrolysate than that from other chemical treated fruit wastes (Gashaw 2014). Unfortunately, the ideal mould type which can be efficiently fermented both C₅ and C₆ sugars has not been yet isolated, nonetheless it cannot ferment the hemicellulose which are converted during pentoses fermentation into toxic furfural (Farias et al 2017). Up-to dated researchers have valorised the ability of ethanologens yeast strains for simultaneous cultivation with another strain with *S. cerevisiae*, this trend became widespread to be effective for fermenting both hexoses (glucose) and pentoses (xylose) sugars for high bioethanol yield which decreased the initial investment and maintenance costs (Yadav et al 2011). Occasionally, the yeast strain of *Kluyveromyces marxianus* is economically advantageous that can metabolize a wide variety of sugars via homo-ethanol pathway, although it requires a higher cells number than *S. cerevisiae* to produce comparable amounts of bioethanol (Widmer et al 2009). As microbial fermentation is an overpower process, special attention is required to control fermentation temperature, so the alcoholic yeast strains resistant to high temperatures are more favourable for bioethanol industry (Fahim 2017). Temperature is an essential parameter for the fermentation process, the ethanol production increases with increasing temperature, the temperatures that allow an optimal growth and better yield of bioethanol are generally range between 20°C and 35°C (Li et al 2010). The theoretical maximum hydrous bioethanol formed after distillation process of fermentation broth was achievable no more than 95.5 wt % ethanol and 4.5% water (Kumar et al 2010). However, the fuel consumption is increasing sharply despite the apparent lack of conventional fossil fuel supplies, only pure bioethanol can be used as a fuel or blended with gasohol as octane boosters (Li et al 2010). The most important bioethanol proper burning benefits that air quality will improve by CO₂ mitigation, bioethanol can also improve the octane number by replace with other additives in

gasoline fuel, gasoline-bioethanol blend provides the highest braking power (Cutzu and Bardi 2017). Also, bioethanol fuel is sulphur free and totally biodegradable, that the incomplete oxidation pi-products are acetaldehyde and acetic acid which are less toxic by comparison with other alcohols (Sharma 2006). Although, the larger scale and world commercial production of biofuels mainly were mostly from essential oil, food, fodder crops at such a global scale would be another challenge for the availability of alternative feedstocks at that many scales (Makkar 2013). Therefore, the cost-effective means for producing bioethanol from four fruit peels (Banana, Orange, Mango and Watermelon), enzymes yielded from co-fungal cultivation carried by *Aspergillus niger* and *Phanerochaete chrysosporium*, followed by co-fermentation of the saccharified sugars using yeast belong to *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* which contributed for bioethanol production in 8 L lab-scale reactor, additional distillation process by adding drying agents increased the bioethanol final purity.

2 Materials and Methods

2.1 Agricultural wastes preparation

Four different fruits peels *Musa acuminata* (Banana), *Mangifera indica* (Mango), *Citrus aurantium* (Orange) and *Citrullus lanatus* (Watermelon) were collected. Minced samples oven dried at 70°C for 48 h and grind to be used later as source of fermentable sugars. Tissues samples were stored in airtight containers in the dark at room temperature until biochemical analysis. The dry weight (DW) was determined by oven dried of 5 grams of each fresh fruit peel at 105°C for not less than 2 hours or to mass constant, the pH was measured by using HI83141 Hanna instruments. Total proteins were measured as (1 ABS = 1 mg.ml⁻¹) by A₅₅₀ biuret direct method by using UV-Vis Spectro (Thermo Fisher Scientific™) (Bradford 1976).

2.2 Microbial strains and culture media

The identified fungus and yeast strains used in this study were *Aspergillus niger* ATCC[®] 64974[™], *Phanerochaete chrysosporium* ATCC[®] 24725[™], *Kluyveromyces marxianus* ATCC[®] 36142[™] and *Saccharomyces cerevisiae* ATCC[®] 9763[™] brought from the national Mircen (Microbiological Resource Center, Ain Shams University). All fungal strains grown on malt extract agar (MEA, *Difco*) to obtain spores. To achieve the fungal behaviour in terms of mycelial interactions and growths rates on various culture media, fungi mixture cultivated on (MEA), potato dextrose agar (PDA, *Difco*) and *Aspergillus* complete medium (ACM, *Difco*) (De Vries et al 2004). This latest culture medium was chosen and supplemented with 5 % wheat brain (Wb) as the sole source of carbon for achievement of fungi behaviours. In the same order, the (ACM) liquid culture medium was chosen and supplemented with 5 % powders of Banana peel (Bp), Watermelon peel (Wp), Orange peel (Op) and Mango peel (Mp), respectively as carbon substrate source for mixed enzymes production. On the other side, laboratory cultures of *S. cerevisiae* and *K. marxianus* strains were routinely maintained on sodium acetate ascospore agar (SAA, *Himedia*) for creating new generation by ascospores germination (McClary et al 1959). The regenerative strains were screened to use as bioethanol co-producers based on (+20 % glucose, xylose fermentative and alcohols tolerance). While the glucose peptone agar medium, (GPA, *Difco*) was used for examining the new yeasts spontaneous mutant selected from the ascospores sexual conjugation. The yeast strains were primarily examined for xylose utilisation, CO₂ emission, ethanol tolerance and productivity using 2 % glucose or/and xylose (1:1 mixture) supplemented peptone broth medium. The optical densities at OD₆₀₀ were used as indicator of growths rates. While the medium used for ethanol production composed of (Yeast extract 2.5 g.l⁻¹, (NH₄)₂SO₄ 2.5 g.l⁻¹, KH₂PO₄ 1 g.l⁻¹ and MgSO₄ 0.5 g.l⁻¹) which later supplied by amounts of filtrated enzymatically produced sugars.

2.3 Cultivation conditions and fermentation setup

For fungi maintenance and spores inoculums preparation, spore production on MEA medium slants usually requires minimum 72 h of growth at 40°C, spores (conidia) suspended in 5 ml of sterile water and then filtrated through sterile glass wool to avoid presence of fungus mycelia. Spores concentration were determined by measuring the absorbance at 650 nm (ABS 1 cm⁻¹ = 5x10⁶ spores.ml⁻¹). Fungi co-enumerated by inoculating 1 ml of diluted spores suspension (5x10⁵ spores.ml⁻¹) with a distance of 3.5 cm or by inoculate the spores mixture in the centre or over the Petri dishes whole surface. For all liquid cultures, fungi grown on 250 ml shaking Erlenmeyer flasks with 40% filling volume, 250 rpm agitation rate and 0.0163 S⁻¹ coefficient volumetric oxygen transfer rate (*k_{LA}*) (Fahim 2017). ACM medium supplemented with 5% of various peels carbon sources, the culture medium inoculated by 5x10⁶ spores.ml⁻¹ and incubated at 40 °C for 96 h. On other side, 8 litre lab-scale fermentor (New Brunswick Scientific MicroFerm MF-114, USA) was used for submerged fermentation (62.5% filling volume, 250 rpm agitation rate and *k_{LA}* 0.0165 S⁻¹) as followed by (Fahim 2017). For enzymes production, ACM medium supplemented with 5% of wheat brain and inoculated by 5x10⁶ spores.ml⁻¹ of mixed fungi, then incubated aerobically at 40°C for 96 h. Culture samples harvested and centrifuged at 10.000 rpm for 10 min, supernatant was then filtrated through 0.045 µm nitrocellulose microbiological membrane filter, the filtrate aliquots culture sample used as degrading enzymes source or stored at -20°C no more than 1 week. For bioethanol production, the culture medium supplemented with 5% powders of (Bp, Mp, Wp and Op) respectively as carbon sources replacements, the culture medium was initially inoculated by 0.5 OD₆₀₀ *S. cerevisiae*, *K. marxianus* or mixed inoculums at 35°C under non aerated condition for 24 h of fermentation period.

2.4 Sugars extraction and oligosaccharides fractionation

The purpose of sugars purification before the oligo-saccharides fractionation was to avoid influence of free sugars on the pectin, hemicellulose, and cellulose determinations. The ethanol extraction method was applied to extract the soluble sugars on each peels tissue sample, five replicates of (50 mg) extracted with 5 ml of ethanol 80% and repeated three times, by boiling at 95°C for 10 min in screw cap glass tubes followed by 2500 rpm centrifugation for 5 min, the collected supernatants combined for sugar analysis. While the rest of the pellets in the tubes were used for starch determination or stored wet at -20°C to analyze the starch later with the hydrolyzed extracted sugars from the oligosaccharides (Cutzu and Bardi 2017). The fruit peels oligosaccharides fractionation process is conducted by the patent application number 420890 published by Szymańska-Chargot and co-workers. 50 grams of minced fruits peels tissues boiled with 300 ml distilled water in 1 L glass piker for 10 min and filtrated to remove simple soluble polysaccharides and phenolic compounds from peels in the aqueous solution. For separating the pectin polysaccharides components, the residual tissues treated by 300 ml of hydrochloric acid (1 M) and magnetically stirred at 85°C for 30 min, and then filtered to collect the pectic polysaccharides compounds in the aqueous solution and the rest of residual bulk was used to extract the hemicellulose polysaccharides compounds by adding 300 ml of sodium hydroxide (1 M). This mixture was magnetically stirred at 85°C for 30 min, and then filtered to collect the hemicelluloses components in the aqueous solution and the rest residual bulk transferred to new glass piker to the next step. Afterwards, the lignin compounds removed by adding 300 ml of sodium hypochlorite (2%) and magnetically stirred at 95°C for 60 min subsequently by transfer the colloidal solution as lignin matter. The end step conducted by collecting the cellulose matter from the rest

bulk amount and washed three times by deionized water until the alkaline traces removed (Ziemiński and Kowalska-Wentel 2017).

2.5 Reduced sugars and oligosaccharides quantifications

Considering the potential use of the end isolation step of cellulose supernatant, the quantification of pectin, hemicelluloses and cellulose contents in fruits peels carried out. The pectic fractions contents performed with colorimetric calibration method of galacturonic acid which transformed into furfural derivatives by reacting with carbazole acid in acidic phase (Orozco et al 2014). The collected pectic residuals rinsed and placed into 200 µl of deionized water followed by gradually addition of 3 ml (H₂SO₄)_{conc} and 100 µl carbazole reagent (0.1% w/v ethanol). The mixer kept for 60 min in the dark at 60°C and cooling through water bath (caution of exothermic reaction), then after development of pink-red colour, the absorption read at wavelength 530 nm were done against the standards curves obtained from galacturonic acid. However, the acid hydrolyzed GFX monomer sugars mixture (glucose, fructose, and xylose) in peels tissue extracts from the oligosaccharides such as hemicelluloses determined by the phenol-sulfuric acid method (Dubois et al 1956). The reaction of 1 ml phenol 2% solution with 0.5 ml of examined sugars solutions, the standard sugars were made by increments concentrations from 50 to 100 µg.ml⁻¹ of the most common sugar mixture with percentage (1:1:1 GFX mixture) or the individual glucose and xylose solutions, respectively. This reaction followed by the quickly and gradually addition of 2.5 ml (H₂SO₄)_{conc} and kept for 10 min in the dark at 20°C in cooling water bath (caution of exothermic reaction), subsequently after development of yellow brown colour, the absorption read at wavelength 490 nm were done against the standards curves obtained from individual sugars or the GFX mixture for total reduced sugars assay.

2.6 Degrading enzymes assays and SDS-PAGE profiles

For the cellulase enzyme activity, the method of filter paper (Whatman no.1) activity was applied (Camassola and Dillon 2012). β -glucosidase activity determined by using 5-carboxy methyl cellulose (10 g.l^{-1}) as carbon replacement source in ACM media (Saddler and Mes-Hartree 1984). Xylanase activity determined under the same conditions by supplying ACM media with (1 ml.l^{-1}) extracted wheat bran solution (Saddler and Mes-Hartree 1984). While the liberated reduced sugars from cellulose as (glucose) and (xylose) from hemicellulose in the latest reaction mixtures measured by the phenol-sulfuric acid methods which used later to determine the extracted monomers sugars from the oligosaccharides, each $1 \mu\text{mol.min}^{-1}.\text{ml}^{-1}$ of released glucose or xylose expressed as one unite of cellulase, β -glucosidase and xylanase enzymes respectively under specified experimental conditions. Protein precipitation carried out with salting technique by adding ammonium sulphate slowly to the cell-free culture (at 75% saturation) with magnetically stirring to complete protein precipitation. The precipitate collected by centrifugation at $10.000 \times g$ for 20 min and then dissolved in 50 mmol phosphate buffer (pH 8.0), dialysed and stored (Switzer et al 1979). For molecular weight determination, the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles for extra-cellular degrading enzymes were preformed, the gel made of (10%) polyacrylamide and 5% stacking gels. After electrophoresis, the gels stained with Coomassie brilliant Blue R-250 staining and overnight de-stained by 45% methanol and 10% acetic acid (Switzer et al 1979).

2.7 Enzymatic hydrolysis and bioethanol production processes

The filtrate culture showing activities of cellulases and xylanases was used for enzymatic hydrolysis processes, the bioreactor supplied with 250 g of fruits peels within 4975 ml

of adjusted pH 5.5 filtrate culture media (buffered by 0.1 M sodium acetate solution), the total hybridisation volume conducted at 50°C for 72 h. By progressing the enzymatic hydrolysis process, collected samples centrifuged at 10.000 rpm for 10 min, supernatant was then filtrated through $0.045 \mu\text{m}$ nitrocellulose microbiological membrane filter, the filtrate aliquots samples used as sugars sources or stored at 5°C no more than 1 month. Samples of the used (Bp, Mp, Wp and Op) substrates chemically analysed, the total of each substrate suspensions used to supplement the production bioethanol medium, respectively as carbon source replacement. 5 litre of culture medium was initially inoculated by 0.5 OD_{600} *S. cerevisiae*, *K. marxianus* or mixed inoculums and mechanically stirred by 150 rpm under non aerated condition at 35°C for 24 h of fermentation period (initial sugars concentration determined). After alcoholic fermentation process, the bioethanol yield determined spectrophotometrically according to the modified chromic acid method developed by (Gupte and Madamwar 1997), 1 ml of fermentation culture broth diluted to 5 ml by distilled water and 5 ml of chromic acid reagent added (34 g $\text{K}_2\text{Cr}_2\text{O}_7$ within 325 ml $\text{H}_2\text{SO}_{4\text{conc}}$ and the total volume adjusted to 1000 ml with distilled water). The screw cap tubes heated in a water bath at 60°C for 20 min. the absorbance of alcohols determined at 600 nm by using absolute ethanol standard curve, after that CO_2 levels can be calculated. For collecting bioethanol from culture broth, the azeotropic distillation process applied at 78.2°C and coupling with addition of 0.5 g.l^{-1} calcium oxide as drying agent during distillation process (Li et al 2010).

3 Results and Discussion

3.1 Fungi co-cultivation

To achieve whether the ability of two fungi for growth together in the same culture and their behaviours, several pour plates inoculated by both fungi as described in the enumeration conditions. Different radial extension rates monitored for the two fungi on MEA

solid culture medium. *A. niger* has the fastest growth fungus, while *P. chrysosporium* has particular moderate growth (**Fig 1-A**). The tested fungi have been the ability to grow on all used types of culture media in this investigation, in this order the (ACM) culture resulted the better and fastest culture growth. This liquid culture was chosen and supplemented with 5% wheat brain as the sole source of carbon for fungi enumeration and enzymes production. Since an earlier study reported that on the wheat brain carbon source has stimulation effect on a broad range of hydrolytic enzymes secretion by several *Aspergillus* types (Meijer et al 2011). The co-cultivation which primarily performed on the separated inoculums and spores mixture poured plates showed the life-style behaviour of two fungi. The separated inoculants a distance away from each other formed colonies with complete edges when they met without any significant forced invasion (non-antagonistic effect) for the other fungus occupied plate area, but *P. chrysosporium* could grow around and within the other fungus (**Fig 1-B**). Interestingly, the spores mixed inoculation in the same spot, there is no sectors containing either fungus could be observed on the plates (**Fig 1-C**) generally, *A. niger* has colonies with a black colour, while *P. chrysosporium* characterized by its white colonies. On other side, no fungus mycelia appeared during the first 20 h of cultivation, after 40 h the hypha of both fungi starting to formation, then quickly spread by *P. Chrysosporium* more than *A. niger* which occupied less area in the presence of the other fungus which covered whole the plate with a white hypha and grown within the other fungus to reach outside its colony. These evidences similar with those experiments which performed by combinations of some fungi genera such as *A. niger*, *A. oryzae*, *P. chrysosporium* and *Magnaporthe grisea*, in all these genera combinations no one of these species showed to out contrary of other fungus genus (Rocha-Meneses et al 2019).

3.2 Yeasts co-fermentation

Approximately (34) of *S. cerevisiae* and (42) *K. marxianus* generated yeasts isolates screened for cultivations tests, the highest CO₂ emission strains during glucose or/and xylose sugars fermentations selected. The alcohol tolerance and the ability to mono sugar or co-carbon sugar utilization conducted for the selected two strains type, data presented in (**Table 1**). *S. cerevisiae* selected strain showed the highest indicators levels of sugar utilization rate and gas emission by 1.12 g.l⁻¹.h⁻¹ and 0.2 mol.l⁻¹ respectively when glucose was the carbon source, despite of it cannot be utilize xylose sugar as carbon source (López-Malo et al 2013). While selected *K. marxianus* strain showed double uses of the glucose and xylose or sugars mixture with moderated levels by 0.68 g.l⁻¹.h⁻¹ and 0.12 mol.l⁻¹ CO₂ emission. It worth to note that, the yeast co-culture showed high levels of glucose sugar utilization rate by 1.26 g.l⁻¹.h⁻¹ and 0.17 mol.l⁻¹ CO₂ emission. Also, they recorded good levels of mixed sugar consummation by 0.83 g.l⁻¹.h⁻¹ and 0.16 mol.l⁻¹ gas emission. Although, the possibility of xylose sugar fermentation recorded by selected strain of *K. marxianus* in single or co-culture condition, which it showed lowest level of sugar consummation and CO₂ emission by 0.55 g.l⁻¹.h⁻¹ and 0.12 mol.l⁻¹, respectively. On other side, it is clear from (**Fig. 2**), that *S. cerevisiae* have a potential for growth and make a high ethanol yield only from glucose by 9.23 g.l⁻¹ at no more than 18 h. While *K. marxianus* showed moderated ethanol yield by 4.91, 5.58 and 6.17 g.l⁻¹ from xylose, mixed sugars, and glucose respectively, with more long fermentation period than the other yeast strain. By tracking the co-fermentation of the two strain showed a good ethanol yield by 7.83 g.l⁻¹ from glucose at no more than 16 h and 7.83 g.l⁻¹ from mixed sugars during 24 h. Also, co-fermentation showed moderated values of ethanol yield from xylose sugar by 5.62 g.l⁻¹ but

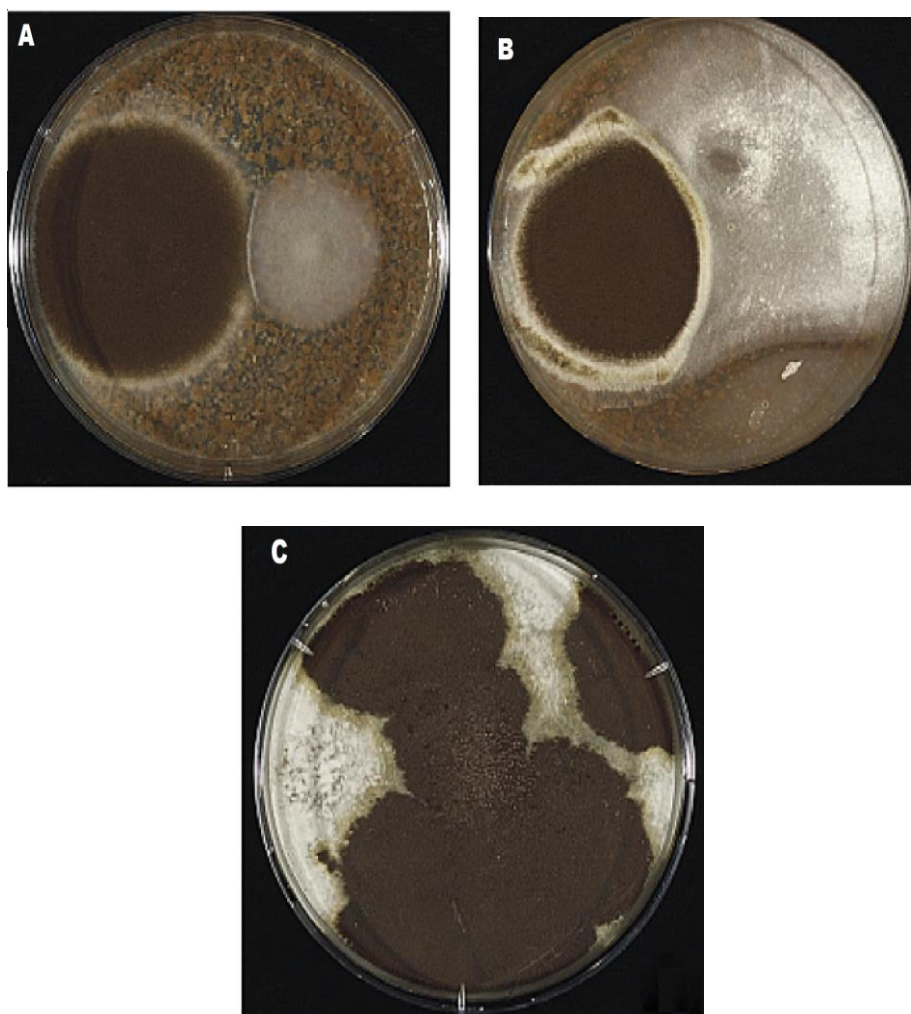


Fig 1. Co-cultivation of *A. niger* and *P. chrysosporium* fungi on solid ACM + 5% Wb by inoculating them separately (A) and after complete growth (B) or as a spore mixture (C)

Table 1. The bioethanol productivity indicators of the selected yeast strains and its co-fermentation

Yeast strains	CO ₂ emission <i>mol.l⁻¹</i>			Sugars utilization rate <i>g.l⁻¹.h⁻¹</i>			Max bioethanol production <i>g.l⁻¹</i>		
	Glucose	Xylose	GX. Mix	Glucose	Xylose	GX. Mix	Glucose	Xylose	GX. Mix
<i>S. cerevisiae</i>	0.2 ± 0.06	0.0 ± 0.0	0.09 ± 0.04*	1.12 ± 0.05	0.0 ± 0.0	1.43 ± 0.08*	9.23 ± 0.04	0.0 ± 0.0	4.21 ± 0.03*
<i>K. marxianus</i>	0.14 ± 0.04	0.11 ± 0.03	0.12 ± 0.08	0.79 ± 0.07	0.57 ± 0.05	0.68 ± 0.06	6.17 ± 0.08	4.91 ± 0.06	5.58 ± 0.06
Co-fermentation	0.17 ± 0.05	0.12 ± 0.07	0.16 ± 0.03	1.26 ± 0.04	0.55 ± 0.03	0.83 ± 0.5	7.83 ± 0.07	5.62 ± 0.04	7.16 ± 0.04

The values are mean of three replicates ± standard deviation.

* Values based on glucose sugar amounts only.

fermentation period rich to more than 36 h by comparing the other single culture xylose fermentation. The previous data strongly referring to the co-fermentation importance by saving fermentation time and increasing the productivity, especially in the presence of xylose sugar which are necessarily available in quantities within treated agro-wastes peels, these results matched with Widmer et al (2009).

3.3 Fruit peels composition

The primarily results of biochemical analyses for four different fruits peels examined are presented in (Table 2). The percentages on dry weight base of total proteins, starch, pectin, lignin, cellulose, and hemicellulose contents investigated, the mango peel showed the highest levels of starch, pectin, hemicellulose, and lignocelluloses components by 13.56%, 11.89%, 14.51% and 51.48% respectively with highest level of lignin content by 17.25% and lowest level of total proteins by 3.89% (Berardini et al 2005). Also, the peel of banana showed high levels of starch, hemicellulose, and lignocelluloses components by 13.27%, 12.93 % and 38.14% respectively with the lowest level of lignin contents by 4.82%, the proteins and pectin contents were in the moderated levels by 5.12% and 11.21%, respectively (Li et al 2010). While the orange peel showed the lowest levels of starch, hemicellulose, and lignocelluloses components by 3.22%, 9.27% and 30.76% respectively with 6.53% of lignin content, but the pectin content recorded the highest level by 16.45%, the proteins levels were moderate by 5.23 %, which close to the report published by (Aravantinos-Zafiris et al 1994). Citrus peels had somewhat lower hemicellulose and lignocellulose content as well. In the same order, the watermelon peel showed the highest level of total proteins by 8.84%. Pectin, hemicellulose and lignocellulose contents were in moderate levels by 11.34%, 11.45% and 33.84% respectively with 9.87% and 11.34% of lignin and pectin

contents, respectively. These results match with the reports of (Fish et al 2009, Li et al 2010) that banana and mango peels could be a rich, worthless cost source of dietary fibres, composed mainly of hemicellulose and lignocellulose polysaccharides (Berardini et al 2005, Li et al 2010). Considering these results, the peels of banana and mango seems to be good alternative of carbon sources suitable for conversion of bioethanol, this preference is due to the respected hexose as well as pentose sugars converted from the good levels of starch, cellulose, and hemicellulose respectively, in addition the ability of tested fungi for reducing the relatively amounts of lignin during the pre-treatment processes and fermentation process, while this view is supported by Ververis et al (2007).

3.4 The co-culture motivation of degrading enzymes

The better set of oligosaccharides degrading enzymes of each fungus specie conducted for achievement the optimum condition of submerged fermentation at the presence of other fungus. The fungi co-cultivation affect investigated by influence the oligosaccharides reduction amounts by degrading exo-enzymes, the selected cultivation conditions were also achieved during fungal species grown together on ACM culture media supplied with wheat bran and compared with the same conditions on single fungus culture, the data presented in (Table 3) illustrated the activities of three oligosaccharide-active enzymes cellulase, β -glucosidase and xylanase enzymes as they previously been remarked to be produced by each fungus strains. Overall, the co-cultivation resulted an increasing in exo-enzymes activities by 5.9, 8.7 and 81.4 $nmol.min^{-1}.ml^{-1}$ for β -glucosidase, cellulase, and xylanase, respectively. Although, *A. niger* showed high enzymes activities compared with *P. chrysosporium*, but the activities of co-cultivation are still higher than to each single fungus culture. These positive co-operation effect between industrial

Table 2. Biochemical analyses of examined fruit peels wastes based on dry weight percentage (% w/w).

fruit peels waste	Protein	Starch	Pectin	Lignocellulose		Hemi-cellulose
				Lignin	Cellulose	
Banana peel	5.12 ± 0.6	13.27 ± 0.3	11.21 ± 0.5	4.82 ± 0.6	33.32 ± 0.3	12.93 ± 0.4
Mango peel	3.89 ± 0.3	13.56 ± 0.7	11.89 ± 0.2	17.25 ± 0.2	34.23 ± 0.5	14.51 ± 0.9
Watermelon peel	8.84 ± 0.5	4.36 ± 0.6	11.34 ± 0.3	9.87 ± 0.4	26.71 ± 0.6	11.45 ± 0.2
Orange peel	5.23 ± 0.9	3.22 ± 0.2	16.45 ± 0.6	6.53 ± 0.7	24.23 ± 0.4	9.27 ± 0.7

The values are mean of three replicates ± standard deviation.

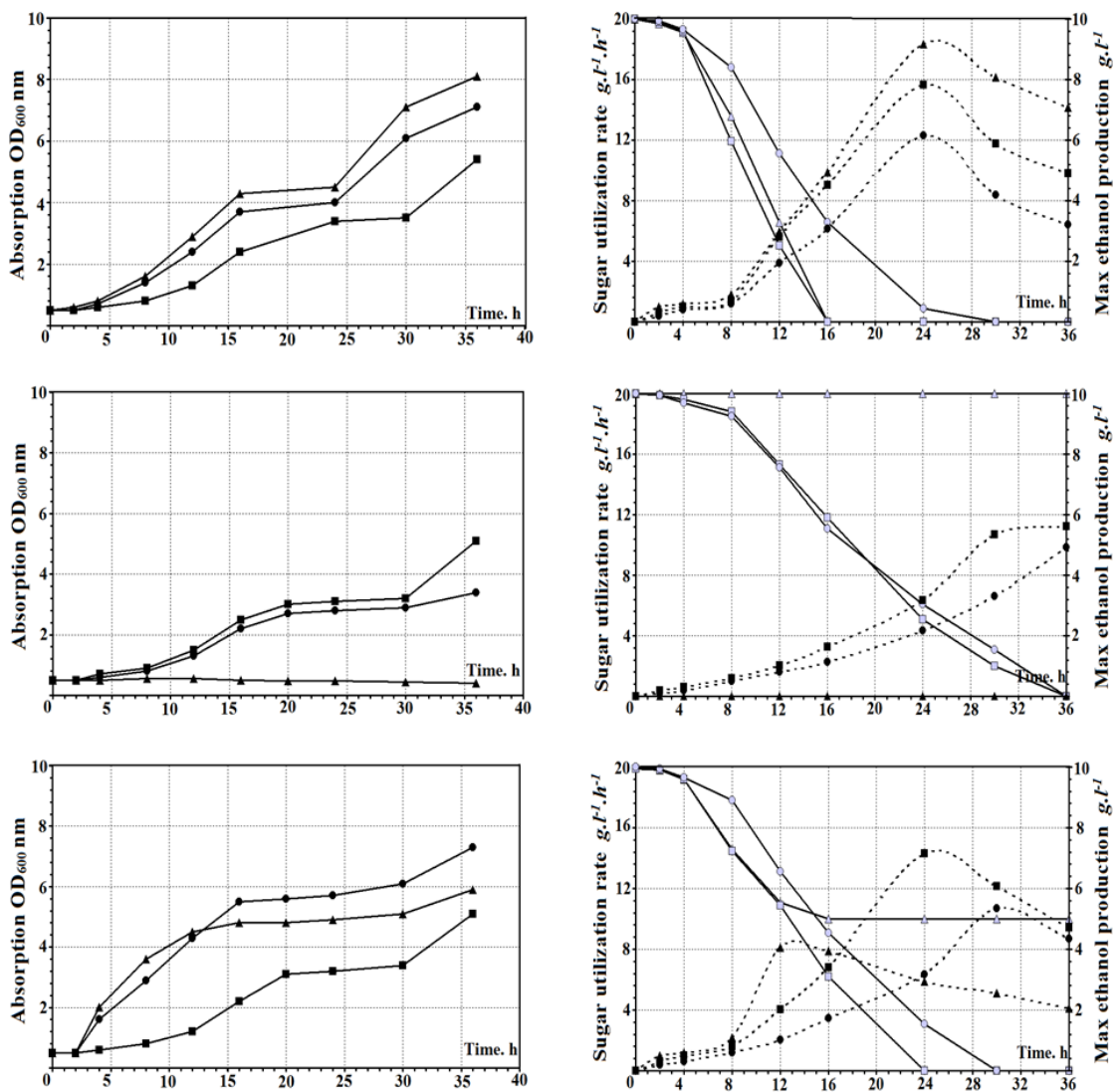


Fig 2. The bioethanol productivity indicators of the selected yeast strains and its co-fermentation of glucose, xylose, and sugars mixture respectively (▲ *S. cerevisiae* ● *K. marxianus* ■ Co-fermentation)

Table 3. Total secreted protein and exo-enzymes activities of *A. niger*, *P. chrysosporium* and its co-cultivation

Fungi strains	Total proteins ($mg.ml^{-1}$)	Exo-Enzyme activities ($\mu mol.min^{-1}.ml^{-1}$)		
		Cellulase	β -glucosidase	Xylanase
<i>A. niger</i>	0.59 ± 0.04	7.9 ± 0.18	4.2 ± 0.12	72.8 ± 0.24
<i>P. chrysosporium</i>	0.42 ± 0.02	6.2 ± 0.15	3.8 ± 0.16	5.3 ± 0.26
Co-cultivation	0.65 ± 0.03	8.7 ± 0.17	5.9 ± 0.17	81.4 ± 0.25

The values are mean of three replicates \pm standard deviation.

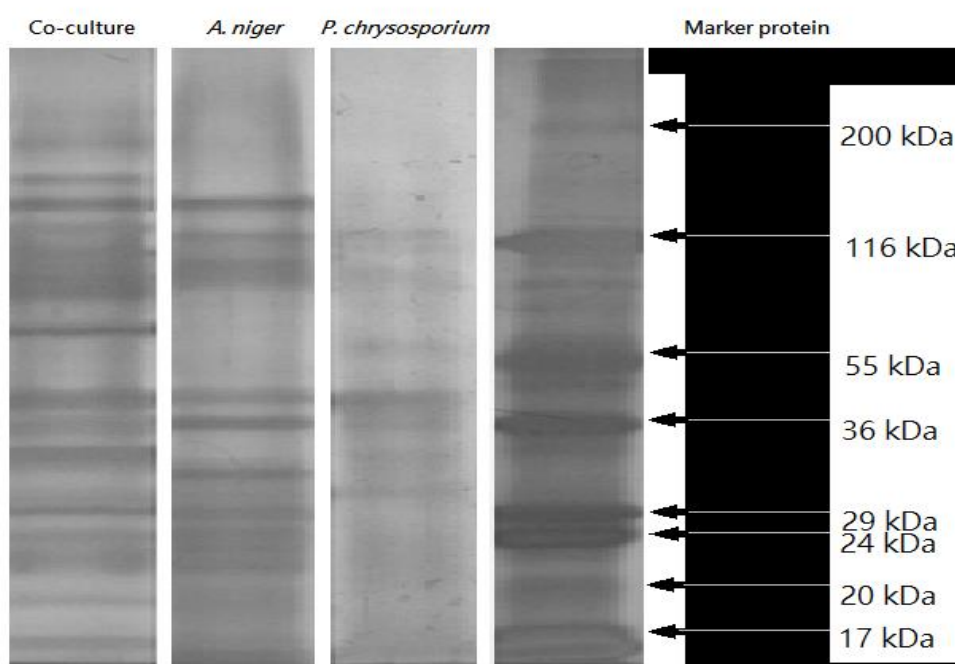


Fig 3. The SDS-PAGE protein profile of *A. niger*, *P. chrysosporium* and its co-cultivation

ascomycete *A. niger* and the wood rot *P. chrysosporium* basidiomycete fungus has been previously recorded by (Meijer et al 2011). The total secreted proteins in co-culture media ($0.65 mg.ml^{-1}$) were also stronger than those resulted by individual culture. The fungi mixed culture does not result a big difference in secreted proteins amounts, but it triggered the stimulation of the specific enzymes. The importance value of the ligninase and hemicellulase enzymes in ameliorate the degrading of lignocellulosic fibers is by observing its synergistic actions along with enzymes of cellulases by increasing swelling and porosity of fibers

and then facilitating its accessibility within it (Hu et al 2011). Also, the mode of action of the ligninase enzyme can formed fragile notches with continuous fissures in the fibers surfaces, while cellulases enzymes complex can begin from these patches, however the enzymes randomly function in synergism harmony (Greene et al 2015). Even though, the co-cultivations enzymes activities were approximately averaging of the two single cultures, but with consecration that only half spores inoculums from every fungus species used by comparing with the mono-cultivations which indicate to the overall activities of fungal co-cultivation and it

can result in specific up-regulation, similar results were recorded for the same fungi strains by (Martinez et al 2004). On the other side, the SDS-PAGE profiles conducted for all culture type (**Fig 3**). Although, the figure illustrated the proteins profile of *A. niger* appeared to similar with the combined fungi proteins, but only fungi combined proteins profile contained proteins not observed in the individual fungus culture. These further proteins are undoubtedly related to the others degrading pectinase and ligninase enzymes, this case was also reported by Hu, Martinez, and they co-workers, that an increase of laccase activity was observed for *A. niger* and *P. chrysosporium* co-culture (Martinez et al 2004). The proteomics studies on the cellulase enzyme have been further confirmed that, several extracellular fungal proteins observed due to the increasing on their synergistic action which were resulted from the release of cell separating exo-enzymes of, carboxymethyl cellulase, cellulase, xylanase, pectinase and other enzymes. Therefore, the hole hydrolase enzyme can be used to design a mixture of enzymes.

3.5 Saccharification and bioethanol production

The process of enzymatic degradation has been followed for achieved the effectually oligosaccharides hydrolyze into fermentable sugars (**Table 4**), the saccharified sugars yields and bioethanol production varied among the type of supplemented peels into production medium. Also, the bioethanol produced depended on the type of inoculated yeast strain. The mango and banana peels showed the highest saccharification of pentose and hexose sugars, the total released fermentable sugars reached to by 27.77 and 21.13 $g.l^{-1}$, respectively. In case of watermelon peels, the released fermentable sugars found to be at moderate level by 19.56 $g.l^{-1}$, while orange peels

produced 15.97 $g.l^{-1}$ at the lowest level of total fermentable sugars. Therefore, the bioethanol yield produced by single *S. cerevisiae* fermentation was the best by 9.19, 6.07, 5.62 and 4.45 $g.l^{-1}$ from saccharified peels of banana, watermelon, mango, and orange, respectively. As expected, the co-fermentation showed an increasing in bioethanol productivity by more than 18% as average percentage for all saccharified fruits peels. The maximum bioethanol productivity obtained from saccharified banana peels by 10.74 $g.l^{-1}$. Although, the mango peels founded to composite a good yield of oligosaccharides which they converted into a good yield of fermentable sugars, but the presence of high percentage of lignin components may resulted a microbial inhibitors derivative as well orange peel. These presented results matched with those obtained by (Reddy et al 2003, Li et al 2010), which they have reported that, the mango peels released good amounts of fermentable sugars, but the ethanol yield was very low by direct fermentation. On other hand, the bioethanol azeotropic separation process coupled by addition of calcium oxide as drying agent which led finally to achievable 95.5 wt % of pure bioethanol. The distillation of bioethanol formed on fermentation broth will lead to produce azeotropic pure ethanol with 95.5 wt % of final purity, to remove the remaining water, duplicate process applied to reach anhydrous ethanol 97.9 wt % (Kumar et al 2010). Among several expanding fruits peels fermentation is well known worldwide, other saccharified peels fermentation is extremely rare, but it is already available due to their sugars rich contents (Reddy et al 2003). Until now, the fruit peels fermentation has mainly been covered for only lignin free residuals due to technical and economic considerations, while a lot of fruit peels residue disposed as waste can become low-cost substrates for biofuel production (Banerjee et al 2010, Lee et al 2014).

Table 4. Total saccharified sugars and bioethanol yields from *S. cerevisiae*, *K. marxianus* and its co-fermentation

Saccharified fruits peels	Total enzymatic released sugars $g.l^{-1}$			Bioethanol $g.l^{-1}$ by yeasts strains		
	Pentose sugars*	Hexose sugars*	Mixed sugars*	<i>S. cerevisiae</i>	<i>K. marxianus</i>	Co-fermentation
Banana	6.08 ± 0.6	22.13 ± 0.2	27.77 ± 0.7	9.19 ± 0.2	7.67 ± 0.7	10.74 ± 0.8
Mango	5.39 ± 0.4	16.26 ± 0.7	21.13 ± 0.3	5.62 ± 0.5	4.89 ± 0.5	6.75 ± 0.7
Watermelon	5.26 ± 0.3	14.47 ± 0.4	19.56 ± 0.4	6.07 ± 0.3	5.36 ± 0.3	7.35 ± 0.5
Orange	4.08 ± 0.7	12.05 ± 0.5	15.97 ± 0.6	4.45 ± 0.5	3.89 ± 0.6	5.31 ± 0.7

The values are mean of three replicates ± standard deviation.

*Pentose, hexose, and mixed sugars (as xylose, glucose and with fructose mixture, respectively).

4 Conclusion

Fruit peels wastes annually accumulate in huge amounts, the most discarded fruit peels were selected to determine their added values, the peels of (Orange, Banana, Mango and Watermelon) were effectively fractionated into their beneficial composition of oligosaccharides. The total proteins, starch, pectin, lignin, celluloses, hemicellulose fractions were detected in terms of dry weights percentages for the studied fruit peels. The highest oligosaccharides levels (62.3%) were in mango peels but with high levels of fermentation inhibitor lignin content. While banana peels also given high oligosaccharides levels (59.52%) with the lowest level of lignin. During the degraded fungal enzymes co-production, no antagonistic phenomenon was observed between the studied co-cultivated fungal strains of *Aspergillus niger* and *Phanerochaete chrysosporium*, that the co-cultivation can improve extracellular enzyme secretion which resulted an increasing in exo-enzymes activities by 5.9, 8.7 and 81.4 $nmol.min^{-1}.ml^{-1}$ for β -glucosidase, cellulase, and xylanase, respectively. The SDS-PAGE protein profiles achieved that, co-cultivation of the two fungi results in improved the relevant exo-enzymes, the combined fungal profile was contained proteins not observed in the fungus monoculture. On other side, bench top lab-scale biostatic reactor was carried out for exo-enzymes production and saccharification. The total saccharified pentose and hexose fermentable sugars from banana, mango, watermelon,

orange peels were 27.77, 21.13, 19.56 and 15.97 $g.l^{-1}$, respectively. Two sexual regenerative yeast strains belong to *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* contributed for co-production of bioethanol. The productivity of bioethanol increased by more than 18% as average percentage for all saccharified peels, most substantial yield was from saccharified banana peels by 10.74 $g.l^{-1}$. Additional distillation process was applied by adding various drying agents, the adding of calcium oxide increased the purity which leads finally to 97.5 wt % of pure ethanol.

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Conflicts of Interest: The authors declare no conflict of interest.

References

- Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB (2011) Biomass pretreatment: fundamentals toward application. *Biotechnol Adv* 29, 675-85.
- Aravantinos-Zafiris G, Oreopoulou V, Tzia C, Thomopoulos C (1994) Fibre fraction from orange peel residues after pectin extraction. *Lwt - Food Sci Technol* 27, 468-471.

- Arya A, Shah A, Sadasivan S (2001) Indoor aeromycoflora of Baroda Museum and deterioration of Egyptian mummy. *Curr Sci* 81, 793-799.
- Banerjee S, Mudliar S, Sen R, Giri B, Satpute D, Chakrabarti T, Pandey RA (2010) Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies. *Biofuel Bioprod Biorefin* 4, 77-93.
- Berardini N, Knödler M, Schieber A, Carle R (2005) Utilization of mango peels as a source of pectin and polyphenolics. *Innov Food Sci Emerg Technol* 6, 442-452.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-54.
- Camassola M, Dillon AJP (2012) Cellulase determination: Modifications to make the filter paper assay easy, fast, practical and efficient. 1: 125. doi:10.4172/scientificreports.125
- Chen H, Fu X (2016) Industrial technologies for bioethanol production from lignocellulosic biomass. *Renew Sust Energ Rev* 57, 468-478.
- Cutzu R, Bardi L (2017) Production of bioethanol from agricultural wastes using residual thermal energy of a cogeneration plant in the distillation phase. *Ferment* 3, 24.
- de Vries RP, Burgers K, van de Vondervoort PJ, Frisvad JC, Samson RA, Visser J (2004) A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. *Appl Environ Microbiol* 70, 3954-9.
- Demirbas A (2011) Competitive liquid biofuels from biomass. *Appl Energ* 88,17-28.
- Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol* 63, 258-66.
- DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal chem* 28, 350-356.
- Fahim S (2017) Production cursors of lipopeptides families by some *Bacillus* spp. *Int J Chem Tech Res* 10, 1096-1103.
- Farias D, Atala DIP, Maugeri F (2017) Improving bioethanol production by *Scheffersomyces stipitis* using retentostat extractive fermentation at high xylose concentration. *Biochem Eng J* 121, 171-180.
- Fish WW, Bruton BD, Russo VM (2009) Watermelon juice: a promising feedstock supplement, diluent, and nitrogen supplement for ethanol biofuel production. *Biotechnol Biofuels* 2,18.
- Gashaw A (2014) Bioethanol Production from Fruit Wastes and Factors Affecting Its Fabrication. *Int J Chem Natural Sci* 2, 32-140.
- Greene ER, Himmel ME, Beckham GT, Tan Z (2015) Glycosylation of Cellulases: Engineering Better Enzymes for Biofuels. *Adv Carbohydr Chem Biochem* 72, 63-112.
- Gupte A, Madamwar D (1997) Solid State Fermentation of Lignocellulosic Waste for Cellulase and β -Glucosidase Production by Cocultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus*. *Biotechnol prog* 13, 166-169.
- Hu HL, van den Brink J, Gruben BS, Wösten HAB, Gu JD, de Vries RP (2011) Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi. *Int Biodeter Biodegr* 65, 248-252.
- Kumar S, Singh N, Prasad R (2010) Anhydrous ethanol: A renewable source of energy. *Renew Sust Energ Rev* 14, 1830-1844.
- Lee S, Speight JG, Loyalka SK (2014) Handbook of alternative fuel technologies, 2^{ed} ed, Boca Raton, CRC Press, <https://doi.org/10.1201/b17157>
- Li K, Fu S, Zhan H, Zhan Y, Lucia L (2010) Analysis of the chemical composition and morphological structure of banana pseudostem. *BioResources* 5, 576-585.

- López-Malo M, Querol A, Guillamon JM (2013) Metabolomic comparison of *Saccharomyces cerevisiae* and the cryotolerant species *S. bayanus* var. *uvarum* and *S. kudriavzevii* during wine fermentation at low temperature. *PLoS One* 8, e60135.
- Makkar HP (2013) Biofuel co-products as livestock feed-opportunities and challenges: processing. *AFMA Matrix* 22, 23-25.
- Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsarm D (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat Biotechnol* 22, 695-700
- McClary DO, Nulty WL, Miller GR (1959) Effect of Potassium Versus Sodium in the Sporulation of *Saccharomyces*. *J Bacteriol* 78, 362-8.
- Meijer M, Houbraken JA, Dalhuijsen S, Samson RA, de Vries RP (2011) Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. *Stud Mycol* 69, 19-30.
- Oberoi HS, Vadlani PV, Madl RL, Saida L, Abeykoon JP (2010) Ethanol production from orange peels: two-stage hydrolysis and fermentation studies using optimized parameters through experimental design. *J Agric Food Chem* 58, 3422-9.
- Orozco RS, Hernández PB, Morales GR, Núñez FU, Villafuerte JO, Lugo VL, Ramírez NF, Díaz CEB, Vázquez PC (2014) Characterization of Lignocellulosic Fruit Waste as an Alternative Feedstock for Bioethanol Production. *BioResources* 9, 1873-1885
- Reddy GV, Babu PR, Komaraiah P, Roy KRRM, Kothari IL (2003) Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor-caju*). *Process Biochem* 38, 1457-1462.
- Rocha-Meneses L, Raud M, Orupöld K, Kikas T (2019) Potential of bioethanol production waste for methane recovery. *Energy* 173, 133-139.
- Saddler JN, Mes-Hartree M (1984) The enzymatic hydrolysis and fermentation of pretreated wood substrates. *Biotechnol Adv* 2, 161-81.
- Sharma DK (2006) Bioprospecting for drug research and functional foods for the prevention of diseases-Role of flavonoids in drug development. *JSIR* 65, 391-401.
- Switzer RC, Merrill CR, Shifrin S (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem* 98, 231-7.
- Verma P, Madamwar D (2002) Production of ligninolytic enzymes for dye decolorization by cocultivation of white-rot fungi *Pleurotus ostreatus* and *phanerochaete chrysosporium* under solid-state fermentation. *Appl Biochem Biotechnol* 102-103
- Ververis C, Georghiou K, Danielidis D, Hatzinikolaou DG, Santas P, Santas R, Corleti V (2007) Cellulose, hemicelluloses, lignin and ash content of some organic materials and their suitability for use as paper pulp supplements. *Bioresour Technol* 98, 296-301.
- Widmer WW, Narciso JA, Grohmann K, Wilkins MR (2009) Simultaneous saccharification and fermentation of orange processing waste to ethanol using *Kluyveromyces marxianus*. *Biol Eng Trans* 2, 17-29.
- Yadav KS, Naseeruddin S, Prashanthi GS, Sateesh L, Rao LV (2011) Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. *Bioresour Technol* 102, 6473-8.
- Ziemiński K, Kowalska-Wentel M (2017) Effect of Different Sugar Beet Pulp Pretreatments on Biogas Production Efficiency. *Appl Biochem Biotechnol* 181, 1211-1227.



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