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MiniReview

Bioconversions of maize residues to value-added coproducts using yeast-like fungi[1]

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Abstract

Agricultural residues are abundant potential feedstocks for bioconversions to industrial fuels and chemicals. Every bushel of maize (approximately 25 kg) processed for sweeteners, oil, or ethanol generates nearly 7 kg of protein- and fiber-rich residues. Currently these materials are sold for very low returns as animal feed ingredients. Yeast-like fungi are promising biocatalysts for conversions of agricultural residues. Although corn fiber (pericarp) arabinoxylan is resistant to digestion by commercially available enzymes, a crude mixture of enzymes from the yeast-like fungus *Aureobasidium* partially saccharifies corn fiber without chemical pretreatment. Sugars derived from corn fiber can be converted to ethanol or other valuable products using a variety of naturally occurring or recombinant yeasts. Examples are presented of *Pichia guilliermondii* strains for the conversion of corn fiber hydrolysates to the alternative sweetener xylitol. Corn-based fuel ethanol production also generates enormous volumes of low-value stillage residues. These nutritionally rich materials are prospective substrates for numerous yeast fermentations. Strains of *Aureobasidium* and the red yeast *Phaffia rhodozyma* utilize stillage residues for production of the polysaccharide pullulan and the carotenoid astaxanthin, respectively.
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Keywords: Astaxanthin; *Aureobasidium*; Corn fiber; *Phaffia rhodozyma*; Pullulan; Xylitol

1. Introduction

Agricultural residues are attractive as potential alternative feedstocks for fuel and chemical production because of their availability, low value and rich lignocellulosic composition. Residues include corn stover, corn fiber and bran, rice and wheat straws, sugarcane bagasse, and sugar beet and fruit pulps. As part of its National Program 307, Bioenergy and Energy Alternatives, the United States Department of Agriculture has targeted corn fiber because of its ready availability and low value (http://www.nps.ars.usda.gov). Program goals include the enzymatic saccharification of corn fiber and the development of new value-added coproducts from conventional fuel ethanol residues.

Corn fiber is a byproduct of the wet-milling process [1]. Briefly, kernels are steeped and then milled to remove the fiber, gluten and germ fractions. The remaining starch can be saccharified enzymatically to produce corn syrup, which then can be converted to high-fructose corn syrup or fermented to ethanol or other products. Stillage residues from ethanol production are folded into the corn fiber to produce corn gluten feed, sold as low-value cattle feed. Nearly half of all fuel ethanol production in the USA employs a wet-milling process, utilizing more than 300 million bushels of corn in 2001 (National Corn Growers Association, http://www.ncga.com/03world/main/index.html). In addition, more than three times that amount of corn is wet-milled annually for production of starch and sweeteners. New coproducts from corn fiber and corn gluten feed thus could improve the economics of fuel ethanol production and add value to the corn processing industry.

Saccharomyces is the premier industrial organism for production of ethanol from glucose, the primary constitu-

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ent of both starch and cellulose. Other yeasts, particularly *Candida* and *Pichia* species (and now recombinant organisms), are able to utilize the abundant pentose sugars derived from hemicellulose for production of ethanol and sugar alcohols. Yeast-like fungi such as *Aureobasidium* are promising candidates for saccharification of biomass. Numerous valuable bioproducts, including microbial gums, vitamins and carotenoids, could be produced from agricultural residues through fermentations by yeast-like fungi. This brief review outlines some of the potential uses of yeast-like fungi in bioconversions of maize residues.

2. Saccharification of corn fiber

Corn fiber is primarily composed of the outer kernel covering or seed pericarp, along with adherent starch. Compositional analyses of corn fiber vary considerably according to the source of the material and methods of analysis. Generally, corn fiber has been reported to include 30–50% arabinoxylan and 15–20% cellulose [2]. Adherent starch levels vary by production facility and on a day-to-day basis, but can be 10–25% or greater. Corn fiber is fairly low in protein (11–12%) and generally believed to be extremely low in lignin.

There is a great deal of interest in obtaining fermentable sugars from corn fiber for conversion to value-added coproducts. Chemical hydrolysis of biomass is efficient and inexpensive, but generates waste products and fermentation inhibitors. Enzymatic saccharification is attractive as a biocompatible, environmentally responsible alternative. However, enzymes may be prohibitively expensive. Furthermore, although the starch and cellulose associated with corn fiber can be hydrolyzed by conventional enzymes, corn fiber xylan has proven to be a recalcitrant substrate. Commercially available xylanases (most from *Trichoderma* and *Aspergillus*) appear to be largely ineffective against corn fiber xylan. Hespell et al. have reported that corn fiber is partially saccharified by commercial enzymes only after pretreatment [3]. A mixture of commercial amylase, glucoamylase, cellulase, cellobiase, and xylanase (at 28 IU xylanase per g fiber) releases 18% of xylose and 40% of arabinose after 72 h at 50°C from corn fiber pretreated by the ammonia-fiber explosion process [3]. Saha and Bothast have tested a mixture of five commercial enzyme preparations at a ratio of 205 IU xylanase per g of corn fiber [4]. After 87 h at 45°C, pH 5.0, this mixture released about 20% of arabinose from arabinoxylan, but no xylose. The further addition of accessory enzymes and high doses of xylanase (not specified) reportedly did not enhance the release of xylose. After an extensive pretreatment with alkali (121°C for 3 h), enzymes released about 18% of xylose and 37% of arabinose after 90 h of digestion [4].

Xylan is a complex heteropolysaccharide with a backbone of β-1,4-linked xylose and various side chains and modifications depending on the plant and tissue source. Endo-β-1,4-xylanase is the primary enzyme that attacks the backbone structure. However, a suite of auxiliary enzymes may be needed to make the backbone accessible, including β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and feruloyl esterase [5]. Corn fiber arabinoxylan also appears to have a complex cross-linked structure [6]. Thus, it is possible that commercial enzyme preparations lack the right combination of enzymes needed to efficiently saccharify corn fiber. A promising alternative source of xylanolytic enzymes is the yeast-like fungus *Aureobasidium*.

Brightly pigmented, so-called color variant strains of *Aureobasidium* are good sources of endoxylanase with a high specific activity [7–9]. Color variant strains overproduce xylanase at 10–100 times levels of typically pigmented strains. As summarized in Table 1, *Aureobasidium* sp. color variant strain NRRL Y-2311-1 grows well on alkaline hydrogen peroxide (AHP)-pretreated corn fiber and produces endoxylanase and auxiliary activities, amylase, and low levels of cellulase and protease [10]. AHP pretreatment is thought to delignify lignocellulose, disorder cellulose and dissociate xylan [11].

As shown in Fig. 1A, concentrated crude enzyme preparations from *Aureobasidium* release an estimated 70% of xylose and essentially all arabinose and glucose from AHP-pretreated corn fiber during 48 h at 37°C [10]. Furthermore, untreated corn fiber is partially saccharified to yield approximately 42% of xylose and 56% of arabinose (Fig. 1B). This finding is encouraging because pretreatments are costly. However, it should be noted that optimal saccharification results require high doses of *Aureobasidium* enzymes (800–3000 IU xylanase per g corn fiber). Although *Aureobasidium* produces substantial levels of xylanase, enzyme costs undoubtedly would be a major factor in the enzymatic saccharification of corn fiber. While endoxylanase itself may not be the limiting enzyme activity, experiments to augment *Aureobasidium* preparations with complementary enzymes thus far have resulted in only modest improvements in corn fiber saccharification (unpublished observations).

3. Fermentations of maize residues to value-added coproducts

3.1. Ethanol

Mixed sugars derived from corn fiber and other biomass sources are potential substrates for fermentation to a variety of valuable products, including amino acids, vitamins, biopolymers, carotenoids, enzymes, and organic acids. Of particular interest is ethanol. It has been estimated that utilization of the corn fiber fraction would increase ethanol yields by about 10% [12]. However, conventional

Table 1
Enzyme activities in culture supernatants of *Aureobasidium* sp. strain NRRL Y-2311-1[a]

Carbon source	Enzyme activities						
	Xylanase (IU ml^{-1})	pNP-Xylosidase (IU ml^{-1})	pNP-Arabinosidase (IU ml^{-1})	pNP-Acetyl esterase (IU ml^{-1})	Amylase (IU ml^{-1})	CM-Cellulase (IU ml^{-1})	Protease (U ml^{-1})
Oat spelt arabinoxylan	294 ± 15	0.001 ± 0.000	0.011 ± < 0.001	1.00 ± 0.03	0.012 ± 0.001	0.031 ± 0.002	0.045 ± 0.003
Untreated corn fiber	8 ± 1	0.022 ± 0.003	0.003 ± < 0.001	0.13 ± < 0.01	0.116 ± 0.006	0.030 ± 0.001	< 0.001
AHP-pretreated corn fiber	90 ± 23	0.046 ± 0.011	0.007 ± 0.001	0.69 ± 0.15	0.63 ± 0.12	0.055 ± 0.004	0.057 ± 0.019

[a]Adapted from [10]. Cultures were grown for 3 days under aerobic conditions at 28°C in a defined basal medium containing the indicated carbon sources at 1.0% (w/v).

Saccharomyces cerevisiae is unable to ferment the pentose sugars that comprise an appreciable fraction of corn fiber hydrolysates.

The first yeast identified as producing ethanol from xylose was *Pachysolen tannophilus* strain NRRL Y-2460 [13,14]. It was subsequently recognized that a number of yeasts have some capacity to carry out this conversion [15–17]. Promising species include *Candida shehatae* [16–19] and *Pichia stipitis* [16,20,21]. Ethanol yields of up to approximately 0.3–0.5 g g^{-1} xylose have been reported. However, all of these yeasts require aerobic growth conditions, and maximal productivity rates (often 0.3–0.4 g l^{-1} h^{-1}) are considered to be disappointing [22]. Arabinose is another important component of corn fiber xylan. Many yeasts convert arabinose to arabinitol, however, none thus far has been identified that produces significant amounts of ethanol from arabinose [15,23]. Although it is conceivable that a naturally occurring yeast will yet be discovered that efficiently ferments mixtures of glucose and pentoses to ethanol, a more promising approach appears to be the rational engineering of conventional microorganisms. Recombinant strains of *Saccharomyces* have been constructed for the conversion of xylose to ethanol [24–30], and recently efforts have begun to introduce genes for the utilization of arabinose [31,32]. A great deal of work also has been done to develop recombinant bacteria for fermentation of glucose, xylose and arabinose to ethanol [33–36]. Recombinant organisms generally show ethanol yields of up to 0.3–0.5 g (g corn fiber sugars)$^{-1}$, with maximal productivity rates of 1.0–1.6 g l^{-1} h^{-1} [35,37,38]. Progress continues towards making these organisms practical industrial biocatalysts. An ambitious ultimate goal might be the construction of *Saccharomyces* strains that contain all of the genes necessary to ferment lignocellulose directly to ethanol.

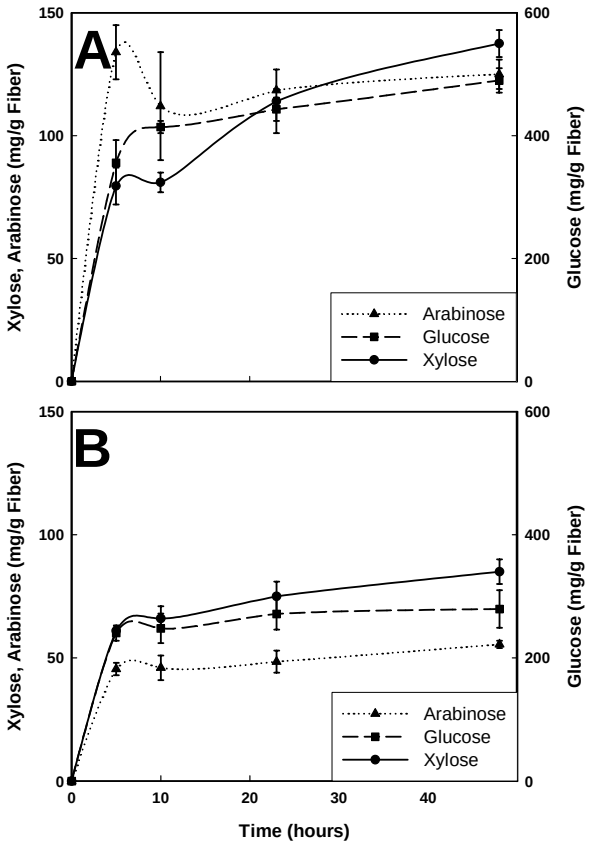

Fig. 1. Time-course of corn fiber saccharification by enzymes from *Aureobasidium* sp. strain NRRL Y-2311-1. Adapted from [10]. Corn fiber suspensions (1.0% w/v) in 50 mM sodium acetate, pH 5.0, were treated at 37°C with concentrated crude enzymes at 3.0 IU (mg corn fiber)$^{-1}$. A: AHP-pretreated corn fiber. B: Untreated corn fiber.

3.2. Xylitol

Another possible fate for xylose is xylitol. Xylitol is a sugar alcohol derivative of xylose, valuable as a sugar substitute [39]. Xylitol is equivalent to sucrose in sweetness, but unlike sucrose it is anticariogenic and metabolized by an insulin-independent pathway. Because it has a significant negative heat of solution, xylitol is especially useful in mints, candies and toothpaste. Xylitol is conventionally produced by a chemical process from birch wood chips and is relatively expensive at about \$7 kg^{-1}. It has been suggested that a bioconversion process could offer a more economical alternative.

Numerous yeasts convert xylose to xylitol, particularly including species of *Pichia* and *Candida* [40–45]. Naturally occurring strains have been reported to produce xylitol under aerobic or semi-anaerobic conditions in yields of up to 0.56–0.74 g g^{-1} xylose with productivity rates of 0.2–0.5 g l^{-1} h^{-1} [43,46–49]. Recombinant strains of *S. cerevisiae* containing the xylose reductase gene from *P. stipitis* or *C. shehatae* convert xylose to xylitol in nearly

Table 2
Growth of *P. guilliermondii* strains on xylose and accumulation of xylitol and riboflavin[a]

Strain	Equivalent number(s)	Isolation source	Isolation site	Growth (cells × 10^9 ml^{-1})	Maximal xylitol (mg ml^{-1})	Final xylitol (mg ml^{-1})	Riboflavin (μg ml^{-1})
NRRL Y-488	ATCC 9058 CBS 2021 IFO 1062	Milk	Unknown	6.1 ± 1.2	3.6 ± 0.3	< 0.05	9.3 ± 1.0
NRRL Y-2075	ATCC 46036 CBS 2030	Frass	Illinois, USA	7.0 ± 0.3	1.4 ± 0.1	< 0.05	11.9 ± 0.7
NRRL Y-2076	ATCC 58070 CBS 2031	Fermentation contaminant	Argentina	7.3 ± 1.5	1.6 ± 0.1	< 0.05	14.4 ± 1.2
NRRL Y-2082	CBS 2082 IFO 0961	Clinical isolate	Delft, The Netherlands	7.0 ± 2.4	1.4 ± 0.1	0.8 ± 0.1	0.9 ± 0.4
NRRL Y-2085	CBS 2086	Air	Lisbon, Portugal	7.2 ± 0.2	1.4 ± 0.3	< 0.05	11.8 ± 5.2
NRRL Y-7572	ATCC 26547 CBS 6557	Fermented food	Chiapas, Mexico	5.2 ± 0.2	3.0 ± 1.1	2.3 ± 0.2	0.5 ± 0.1
NRRL Y-12723	None known	Corn	Georgia, USA	7.3 ± 1.4	7.1 ± 1.1	4.5 ± 0.1	3.1 ± 0.8

[a]Adapted from [55]. Cultures were grown for 95 h under semi-aerobic conditions at 28°C in 2.0% xylose.

stoichiometric 1:1 yields with productivity rates of approximately 0.6–1.0 g l^{-1} h^{-1} [50–54]. However, these recombinant strains require cosubstrates such as glucose for growth and cofactor regeneration. At the same time, high levels of glucose block xylose transport in *Saccharomyces*.

Among naturally occurring yeasts that produce xylitol from xylose, strain variability appears to be an important factor. As summarized in Table 2, *Pichia guilliermondii* strains from diverse sources exhibit maximal xylitol yields that vary considerably on a strain-specific basis [55]. Furthermore, strains fall into two general classes. One class immediately consumes xylitol as soon as xylose is depleted, while a second class only slowly and partially utilizes xylitol, providing a convenient production plateau [55]. Since all of the strains are able to utilize xylitol as a sole carbon source, this re-utilization phenomenon may have to do with cofactor balances [20]. Unexpectedly, strains that re-utilize xylitol also secrete riboflavin, another potential coproduct, during the re-utilization phase [55].

A common feature of xylitol-producing yeasts is that they are subject to glucose repression [44]. Characteristically, *P. guilliermondii* strain NRRL Y-12723 converts xylose to xylitol, and converts a mixture of xylose and arabinose to xylitol and arabinitol [55,56]. However, if glucose is also present, as in corn fiber hydrolysates, the strain preferentially utilizes glucose and only slowly metabolizes xylose and arabinose with little accumulation of sugar alcohols (Fig. 2A) [56]. As one approach to this problem, a two-stage fermentation process has been developed in which glucose-repressed cells are removed from the culture as soon as glucose is depleted and replaced with cells grown on xylose. As shown in Fig. 2B, this method restores xylitol production [56]. In practice this scheme might be realized using tandem immobilized cell columns. Because xylitol is sequentially produced before arabinitol, careful control of the process could be used to bias product yields in favor of xylitol.

3.3. Pullulan

Although a great deal of research has been devoted to bioconversions of corn fiber and other lignocellulosic residues, much less work has been reported on the development of value-added coproducts from corn-based fuel ethanol stillage. The initial or thin stillage (TS) from fuel ethanol distillation is concentrated by evaporation to form corn condensed distiller's solubles (CCDS) which is combined with corn fiber to form corn gluten feed [2]. CCDS contains about 18% protein, a major value for animal feeds, and about 20% carbohydrate, including starch oligosaccharides that escaped saccharification. CCDS also contains growth factors known to include vitamins and peptides. The composition of stillage residues thus resembles a recipe for a fermentation medium. Stillage residues consequently have been tested as substrates for production of the valuable bioproducts pullulan and astaxanthin.

Pullulan is a unique homoglucan often described as a polymer of α-1,6-linked maltotriose, produced by certain strains of the yeast-like fungus *Aureobasidium* [57]. Pullulan has distinctive film- and fiber-forming properties that have been exploited for edible films and related food products. Emerging pharmaceutical applications for pullulan may provide expanded markets. For example, novel oral-care strips based on pullulan films are now commercially available. Pullulan is produced by Hayashibara Biochemical Laboratories of Okayama and wholesales at about \$20–25 kg^{-1} [57].

Aureobasidium sp. color variant strain NRRL Y-12974 produces pullulan with little of the melanin contamination characteristic of typically pigmented isolates [58]. This strain grows well on basal medium containing either corn fiber or CCDS as a carbon source, but not on TS [59]. Polysaccharide yields are similar from CCDS and soluble starch, although higher yields are obtained from glucose or maltose (Table 3). Polysaccharides from both

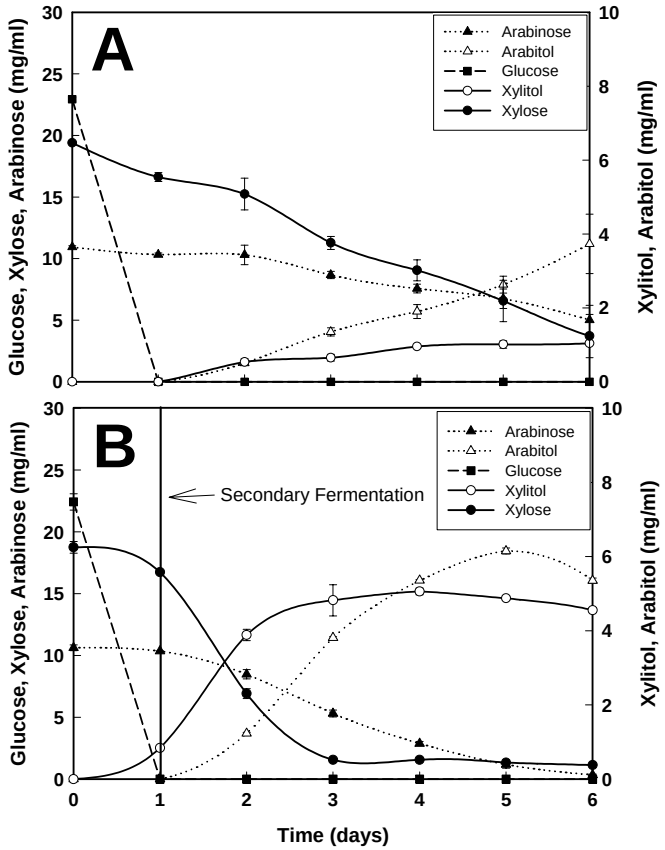

Fig. 2. Fermentations of deionized corn fiber hydrolysate by *P. guilliermondii* strain NRRL Y-12723. Adapted from [56]. Corn fiber was hydrolyzed by treatment with 4.5 ml of 1.0% (v/v) sulfuric acid per g corn fiber for 1 h at 121°C. Hydrolysates were neutralized using calcium hydroxide, clarified by centrifugation and deionized using Bio-Rad AG 501 X-8 (D) mixed-bed resin, then diluted 1:2 into 2× basal growth medium (no additional carbon source). Cultures were grown under semi-aerobic conditions at 28°C. A: Single-stage fermentation. B: Two-stage fermentation. After 24 h, cells were removed by centrifugation and replaced with cells harvested from parallel cultures grown on 2.0% (w/v) xylose.

soluble starch and CCDS cultures also exhibit a lower pullulan purity as judged by pullulanase assay (Table 3). Nevertheless, CCDS is an extremely inexpensive feedstock, and culture optimization might improve pullulan yields and purity. Furthermore, some *Aureobasidium* strains prefer starch oligosaccharides for pullulan production, and commercial production reportedly uses starch hydrolysates of dextrose equivalent 40–50 [57]. Ethanol produced on site with pullulan could be used to precipitate the polysaccharide from cultures and then be cycled back for redistillation.

3.4. Astaxanthin

Astaxanthin is the carotenoid pigment that gives salmon their characteristic color [60]. The pigment is important for consumer acceptance and also may have health and flavor benefits. Salmon cannot synthesize this pigment but must consume it in their diet. For farm-raised salmon, astaxanthin is an expensive feed supplement. The red yeast *Phaffia rhodozyma* is a natural source of astaxanthin that has been commercially developed as an aquaculture feed supplement [60].

Table 3
Growth and polysaccharide yields from *Aureobasidium* sp. strain NRRL Y-12974[a]

Substrate	Growth (cells ml^{-1})	Polysaccharide (g l^{-1})	Pullulanase sensitivity (%)
Glucose	$3.3 \pm 0.3 \times 10^8$	10.1 ± 1.3	$81 \pm 5\%$
Maltose	$2.9 \pm 0.3 \times 10^8$	12.5 ± 1.8	$65 \pm 3\%$
Soluble starch	$3.8 \pm 0.4 \times 10^8$	5.4 ± 0.8	$39 \pm 7\%$
Corn fiber	$5.2 \pm 1.3 \times 10^8$	0.9 ± 0.1	$21 \pm 9\%$
CCDS	$5.8 \pm 3.3 \times 10^8$	4.5 ± 0.2	$28 \pm 12\%$

[a]Adapted from [59]. Cultures were grown aerobically in a defined basal medium containing the indicated growth substrates for 9 days at 28°C. Substrates were 2.0% (w/v) in defined basal medium except for CCDS which was 10% (wet w/v).

Table 4
Growth and carotenoid production by *P. rhodozyma* strains[a]

Strain	Equivalent	Growth (mg yeast ml^{-1})				Carotenoid (μg ml^{-1})			
		YM	CGF	CCDS	TS	YM	CGF	CCDS	TS
NRRL Y-10921	UCD 67-210	4.1 ± 1.6	6.6 ± 0.5	6.6 ± 0.1	6.8 ± 0.4	1.7 ± 0.3	0.7 ± 0.1	1.2 ± 0.1	1.9 ± 0.2
NRRL Y-10922	UCD 76-18	7.1 ± 0.3	6.0 ± 0.5	9.2 ± 0.1	12.2 ± 0.1	3.0 ± 0.2	1.2 ± 0.1	3.1 ± 0.1	3.2 ± 0.7
NRRL Y-17268	BKM Y-2059	4.1 ± 0.9	8.1 ± 0.2	7.5 ± 0.1	10.1 ± 0.7	1.9 ± 0.1	1.5 ± 0.4	2.4 ± 0.2	2.8 ± 0.5
NRRL Y-17269	BKM Y-2268	7.2 ± 0.2	9.9 ± 0.1	9.3 ± 0.1	12.8 ± 0.1	2.6 ± 0.1	1.7 ± 0.2	2.6 ± 0.1	4.1 ± 0.3
NRRL Y-17270	BKM Y-2273	6.6 ± 2.0	7.9 ± 0.3	7.0 ± 0.9	11.4 ± 2.4	2.6 ± 0.3	1.4 ± 0.1	2.3 ± 0.5	2.0 ± 0.5

CGF = 10% (w/v) corn gluten feed; CCDS = 8% (w/v) corn condensed distiller's solubles; TS = 70% (w/v) thin stillage.
[a]Adapted from [61]. Cultures were aerobic at 20°C for 72 h in a control medium (YM) or in clarified coproducts in distilled water.

Five naturally occurring strains of *P. rhodozyma* have been tested for growth and carotenoid production on a standard laboratory medium (YM) and on media containing only clarified corn residues in distilled water [61]. As shown in Table 4, these strains grow well on corn gluten feed, CCDS, and especially on TS [61]. Moreover, carotenoid yields are comparable on TS and YM. *P. rhodozyma* strain NRRL Y-17269 produced 4.1 μg carotenoid ml^{-1} on TS, equivalent to 0.3 μg mg^{-1} yeast dry weight [61]. This strain subsequently was mutagenized and selected for improved carotenoid production on TS. Mutant strain JB2 produces up to 2.0 μg mg^{-1} yeast dry weight [62].

4. Outlook

Although agricultural residues are plentiful and readily accessible, improved methods are needed for their conversion to fermentable sugars. Yeast-like fungi such as *Aureobasidium* may provide novel enzymes useful in the saccharification of lignocellulose. At the same time, new and improved organisms are needed for the utilization of pentose sugars. Naturally occurring and recombinant yeast strains are promising candidates for conversions of these sugars to ethanol or sugar alcohols. Stillage residues from ethanol production are abundant, nutritionally rich byproducts that have the potential to serve as inexpensive fermentation media for production of polysaccharides and carotenoids by yeasts or yeast-like fungi.

Among numerous other products that might be made by yeast fermentations of agricultural residues are organic acids, amino acids, cell wall fractions, and enzymes such as invertase, β-galactosidase and lipase [63,64]. Yeast cells and extracts also have value as single-cell protein and flavorants in foods and feeds. Recombinant yeast strains could make many additional products, including high-value pharmaceuticals. However, agricultural residues are perhaps most appropriate as fermentation substrates for high-volume, low- to moderate-value products for which feedstock costs or availability are limiting. This is why biomass remains attractive for fuel ethanol production despite technical challenges.

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