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PLANT PATHOLOGY & NEMATOLOGY

Ability of Indigenous Yeasts from Aerial Plant Surfaces to Degrade Sugars from Insect Honeydew

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INTERPRETIVE SUMMARY

Insects such as aphids and whiteflies excrete a sugary liquid when they feed on plants. This sugary liquid, termed honeydew, falls onto surfaces as a sticky residue. The “sap” seen on the windshield of a car parked under a tree is one example. When these insects feed on cotton plants, the honeydew can fall onto cotton bolls and create a sticky residue on the cotton fiber. This sticky cotton poses a serious problem for ginning and milling because sugars foul equipment and cause the lint to stick to machinery. The stickiness reduces the lint quality and results in substantial price penalties to the grower. In some areas of the United States, losses of up to $50 million have occurred in a single season. Bioremediation (the use of a living organism to degrade a problem substance) may offer a solution for cleaning this sticky cotton and restoring quality. Yeasts, a group of fungi inhabiting the leaves and stems of cotton and other plants, are good candidates for degrading the honeydew. Our goal was to characterize yeasts for the ability to degrade some of the key sugars in insect honeydew. All sugars tested could be degraded by many of the 250 yeast strains tested, and a wide range of degradation rates was observed on individual sugars. Many strains could degrade several or all the sugars. These results provide important information on the sugar-degrading ability of the yeasts inhabiting plants and will help scientists identify potential bioremediation agents. In the future, bioremediation may give cotton growers an effective tool for cleaning sticky cotton and avoiding serious losses in quality.

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ABSTRACT

Sticky cotton occurs when late-season whitefly or aphid infestations result in honeydew deposition on the lint. Contamination of cotton lint by insect honeydew poses a serious problem for ginning and processing because sugars foul equipment and cause the lint to stick to machinery. Bioremediation, the degradation of compounds by living organisms, offers a possible method of removing this contamination. The yeasts that colonize aerial plant surfaces are potential sources of bioremediation agents. Our research characterized the indigenous yeast population from the aerial surfaces of cotton and other plants growing in the San Joaquin Valley of California for its ability to utilize some honeydew sugars. Yeast strains collected in the field were tested in the laboratory for growth on sucrose, glucose, fructose, melezitose, trehalose, or cellobiose. All sugars tested could be degraded by many of the 250 yeast strains tested, and a wide range of growth rates of the yeast strains was observed on individual sugars. The mean growth rate was highest on sucrose followed by glucose, fructose, cellobiose, trehalose, and melezitose. The 250 yeast strains were grouped with cluster analysis into five major functional groups on the basis of sugar utilization. Many strains could utilize several or all the sugars, and within a single strain, growth rates on different sugars were correlated. These results provide an important characterization of the carbohydrate utilization capabilities of the indigenous yeast population on plants and indicate the potential for selecting bioremediation strains of yeast from the local yeast population.

Sticky cotton occurs when the cotton aphid (*Aphis gossypii* Glover) or silverleaf whitefly *(Bemisia argentifolii* Bellows and Perring) excrete honeydew

onto the lint while feeding on the foliage. Honeydew is a sugar-rich mixture that varies in composition, depending on the insect that is present (Hendrix et al., 1992). The predominant sugars found on sticky cotton are melezitose ($C_{18}H_{32}O_{16}$), trehalulose ($C_{12}H_{22}O_{11}$), sucrose ($C_{12}H_{22}O_{11}$), fructose ($C_6H_{12}O_6$), glucose ($C_6H_{12}O_6$), and trehalose ($C_{12}H_{22}O_{11}$) (Hendrix et al., 1992; Tarczynski et al., 1992; Brushwood and Perkins, 1995). Unique sugars, such as bemisiose ($C_{18}H_{32}O_{16}$), can also occur (Hendrix and Wei, 1994). The individual sugars in honeydew vary in stickiness, and mixtures of sugars tend to be stickier (Miller et al., 1994).

The contamination of cotton lint by insect honeydew is a serious problem that reduces the price of cotton (*Gossypium hirsutum* L.) by damaging quality. Losses of $30 to $50 million have occurred in a single season. Proper late-season insect management is the best way of avoiding sticky cotton, but when insect control measures fail, lint becomes contaminated with honeydew, creating the need for remedial measures. Various remedial measures have been tried. These measures vary from processing aids sprayed on the lint at ginning or spinning (Perkins, 1986) to the application of enzymes applied to break down the sugars (Hendrix et al., 1993; Henneberry et al., 1997). None of these approaches, however, has been entirely successful. Currently, effective remedial measures are not available to clean contaminated lint.

Bioremediation, the use of living microorganisms to degrade the contaminating sugars, may offer an economical and effective way to reduce the stickiness of lint. This technology would offer a rescue treatment for situations when insect controls fail and aphid or whitefly populations result in lint contamination. Several attempts to use various bacteria for bioremediation met with limited success (Balasubramanya et al., 1985; Heuer and Plaut, 1985). Yeasts may be more successful as bioremediation agents because they have several characteristics, not found in most bacteria, that would be desirable in a bioremediation agent for sticky cotton. Yeasts readily consume sugars, grow over a range of temperature and moisture conditions, and withstand desiccation. Yeasts are also easier to culture than other fungi or bacteria (Barnett, 1976). Moreover, yeasts have already been used successfully to reduce honeydew contamination on cereal crops (Dik et al., 1991).

Successful bioremediation relies on the ability to select strains of yeast with wide enzymatic capabilities, high rates of sugar utilization, and adaptation to field conditions. Although genetic engineering might be useful in developing such strains, the current regulatory climate makes it difficult to gain approval for release of genetically engineered microorganisms. Therefore, our plan was to select strains from the indigenous yeast population present on cotton in the San Joaquin Valley.

The present research was initiated to survey the yeast population associated with cotton and other plants from the San Joaquin Valley of California for its ability to degrade some of the sugars found in insect honeydew. Knowledge of the enzymatic capability in the population, the variation in growth rates of yeasts on sugars, and the occurrence of multiple enzymatic pathways in individual strains would provide important information to determine the feasibility of selecting effective bioremediation agents from the local yeast population.

MATERIALS AND METHODS

Yeast Collection

Yeasts were collected from cotton (*Gossypium hirsutum* L. and *Gossypium barbadense* L.) leaves, lint, and from leaves of alfalfa (*Medicago sativa* L. subsp. *sativa*), blackeye bean [*Vigna unguiculata* (L.) Walp.], and several ornamental species growing in the San Joaquin Valley of California. Leaves, lint from bolls, or lint from recently harvested modules were collected and transported back to the laboratory under refrigeration. Samples were washed in 150 mL of sterile, 0.01 *M* phosphate buffer, pH 7.2, containing 0.01% Tween 80. Washing varied with sample type. Leaf samples were washed at 30°C by shaking for 20 min on an orbital shaker at 250 rpm and then transferred to a 30°C ultrasonic cleaning bath (Model 8852, Cole-Parmer, Vernon Hills, IL) for 10 min. Lint samples were washed by processing in a Stomacher blender (Seward Ltd., London). Resulting suspensions were then plated with a spiral plater (Spiral Biotech, Bethesda, MD) onto a recovery medium consisting of malt agar (Difco

Laboratories, Detroit, MI) acidified to pH 4.5 with sterile 10% lactic acid ($C_3H_6O_3$). Plates were incubated at 28°C for 6 d and then stored at 4°C prior to isolation of yeasts. Individual colonies were isolated from plates, suspended in sterile 0.01 *M* phosphate buffer, and streaked on Difco YM agar (Difco). Pure isolates were increased on YM agar, suspended in filter-sterilized YM broth containing 15% glycerol ($C_3H_8O_3$), and frozen at -80°C until analysis.

Sugar Utilization Testing

Solutions of sucrose, glucose, fructose, melezitose, trehalose, or cellobiose ($C_{12}H_{22}O_{11}$) (5.0 g L^{-1}) were prepared in a solution of Bacto yeast nitrogen base (6.7 g L^{-1}) (Difco), filter sterilized, and then dispensed into 96-well microplates (Fisher Scientific, Pittsburgh, PA). All sugars were obtained from Sigma Chemical (Saint Louis, MO). Two controls, water and the nutrient base without added sugar, were also tested to check for yeast growth on endogenously stored nutrients. Cellobiose, although not known to be present in honeydew, was tested because it is a breakdown product of cellulose, and cellobiose utilization is sometimes associated with cellulase activity in nature.

All strains of yeast were grown on Bacto YM agar for 48 h immediately prior to testing and then suspended in sterile water. The optical density of each suspension was adjusted to 0.25 absorbance units at 580 nm using a spectrophotometer (Spec 20, Milton Roy, Ivyland, PA). Plate wells were inoculated with 100 mL of yeast inoculum and mixed with the previously dispensed 100 mL of sugar solution. Each strain by sugar combination was tested in two replicates. Plates were incubated at 28°C and growth was determined at time zero and at approximately 24-h intervals by measuring absorbance at 630 nm with a microplate reader (Model ELx800UV, Bio-Tek Instruments, Winooski, VT).

Statistics

Comparing growth curves for the 250 strains by eight sugars and control combinations was impractical, so data were reduced by calculating growth rate parameters for each of the 2000 yeast strain by sugar combinations. A regression of absorbance against time was used to calculate a growth rate parameter for each strain by sugar combination. Regression analysis was performed with SAS PROC REG (SAS Institute, Cary, NC). An exponential model using the first three readings (0, 24, 48 h) was used for the growth rate analysis. This time span included the exponential phase of the growth curve. The growth rate was expressed as the change in absorbance units per hour. An analysis of variance for growth rates as influenced by sugar and yeast strain was performed with SAS PROC GLM. Treatment means for sugars and controls were separated using the Tukey-Kramer test.

The pattern of utilization of each sugar was examined with principal component analysis using the Factor Analysis module of Statistica (StatSoft, Tulsa, OK). This approach groups sugars according to similarity in utilization by the 250 yeast strains.

The distributions of growth rates among strains on a given sugar were plotted against the standardized normal values of the rates to produce normal probability plots. The normal probability plot facilitates examination of the distribution and identification of outliers.

Cluster analysis, for the purpose of identifying functional groupings of strains, was preformed using the clustering module of Statistica (StatSoft).

RESULTS AND DISCUSSION

All the sugars evaluated were utilized by many of the 250 strains tested. The nutrient base and the water control did not support growth, indicating that cellular carbon reserves of the yeast cells were insufficient to support growth. Growth of yeast on the six sugars followed three characteristic patterns. The first growth pattern, illustrated by Strain 59 (Fig. 1), was characterized by a wide variation in growth rates on the different sugars, but all sugars supported some growth. The second growth pattern, illustrated by Strain 84 (Fig. 2), was characterized by similar growth on all sugars. The third growth pattern, illustrated by Strain 232 (Fig. 3) was characterized by good growth on some sugars but no detectable growth on other sugars. An exponential growth rate for each of the 250 yeast strains growing on the six sugars and two controls was calculated and the growth rates were used for further analysis.

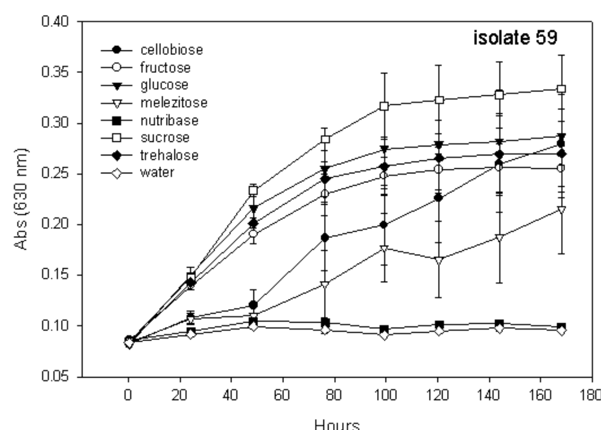

Fig. 1. Growth pattern of some yeasts as illustrated by yeast Strain 59 on six sugars and two controls in which growth occurred on all sugar but at different rates. Growth rate as absorbance units per hour is plotted over time. Points are the average of two replications and standard deviations are indicated.

Table 1. Comparison for yeast growth rates on six sugars and two controls. Mean separation tests conducted using the Tukey-Kramer test (LSD = 0.0011).

Sugar	Mean rate†
sucrose	0.0174 a
glucose	0.0168 a,b
fructose	0.0162 b,c
cellobiose	0.0152 c,d
trehalose	0.0145 d,e
melezitose	0.0141 e
nutrient base	-0.0011 f
water	-0.0038 g

† Means followed by the same letter are not significantly different (Tukey-Kramer test, P = 0.05).

The ANOVA model based on the factors sugar and yeast strain accounted for 84% of the observed variation in growth rates. Exponential growth rates were significantly influenced by sugar and yeast strain. The main effects of strain and sugar were significant ($P < 0.0001$). A detailed examination of the influence of sugar and yeast strain on growth rate is presented below.

Growth Rates as Influenced by the Sugar

When examined across all 250 yeast strains, growth rates on individual sugars varied significantly (Table 1). The mean growth rate was highest on sucrose followed by glucose, fructose, cellobiose, trehalose, and melezitose. This ranking of the sugars

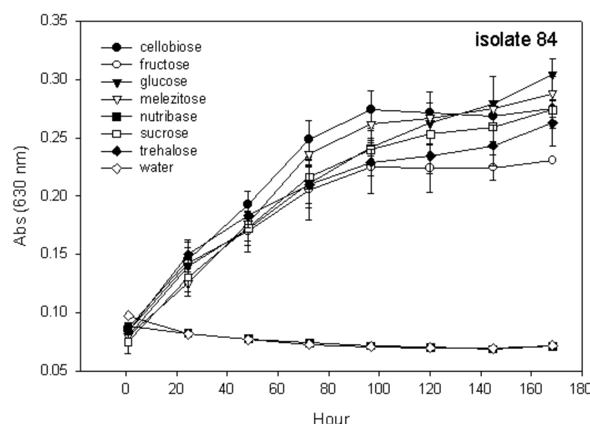

Fig. 2. Growth pattern of some yeasts as illustrated by yeast Strain 84 on six sugars and two controls in which growth occurred on all sugars at similar rates. Growth rate as absorbance units per hour is plotted over time. Points are the average of two replications and standard deviations are indicated. The curve for the nutribase control is partially hidden behind the curve for the water control.

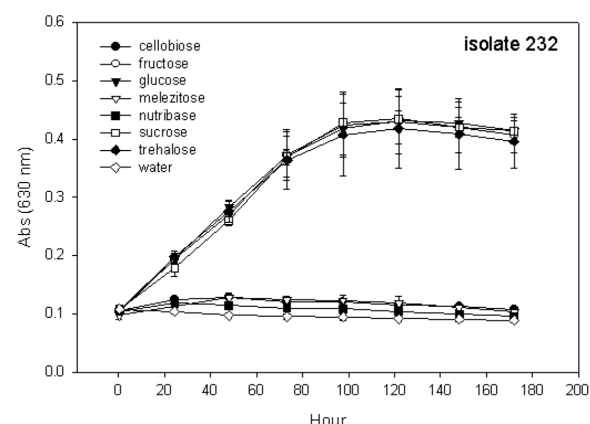

Fig. 3. Growth pattern of some yeasts as illustrated by yeast Strain 232 on six sugars and two controls in which growth did not occur on some sugars (in this case, melezitose and cellobiose). Growth rate as absorbance units per hour is plotted over time. Points are the average of two replications and standard deviations are indicated.

reflects increasing resistance to microbial degradation with melezitose being the most resistant to degradation. Growth rates on nutrient base and water were negative, which indicated that yeast populations declined after inoculation.

When sugars were grouped according to similarity in utilization by yeast strains (Fig. 4), sucrose, fructose, and glucose grouped together indicating that these sugars were utilized at similar rates (either high or low) by the 250 yeast strains

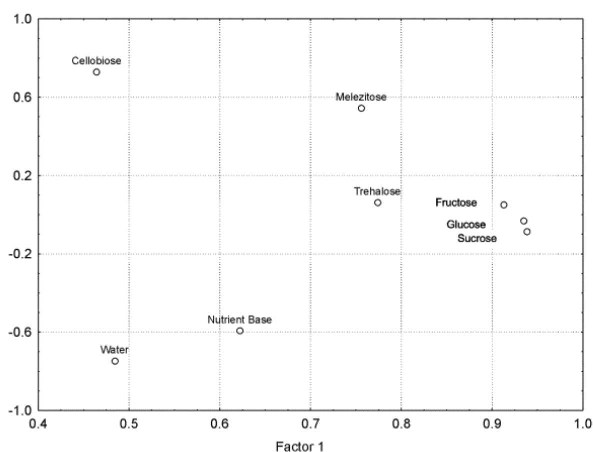

Fig. 4. Comparison of sugar utilization rates based on principal component analysis. Axes are the first and second principal component factor loadings. The two factors accounted for a total of 76% of the variation in growth rates. Sugars grouped together were utilized at similar rates by the population of 250 yeast strains.

evaluated. Melezitose and trehalose were eachlocated near the sucrose, fructose, and glucose cluster. Cellobiose was well removed from the other sugars, indicating a distinctly different pattern of utilization. The water and nutrient base controls were located separate from all of the sugars.

The location of the sugars in Fig. 4 is probably best explained by the structure and enzymology of the sugars (Barnett, 1981). The close grouping of sucrose with glucose and fructose is probably due to the fact that the disaccharide sucrose is readily hydrolyzed to the monosaccharides fructose and glucose. It is also probable that organisms capable of hydrolyzing sucrose can utilize the resulting monosaccharides. Trehalose, a disaccharide, is hydrolyzed to two molecules of glucose and this may explain the location near the group containing glucose, fructose, and sucrose. Melezitose, a trisaccharide, can be hydrolyzed to either turanose

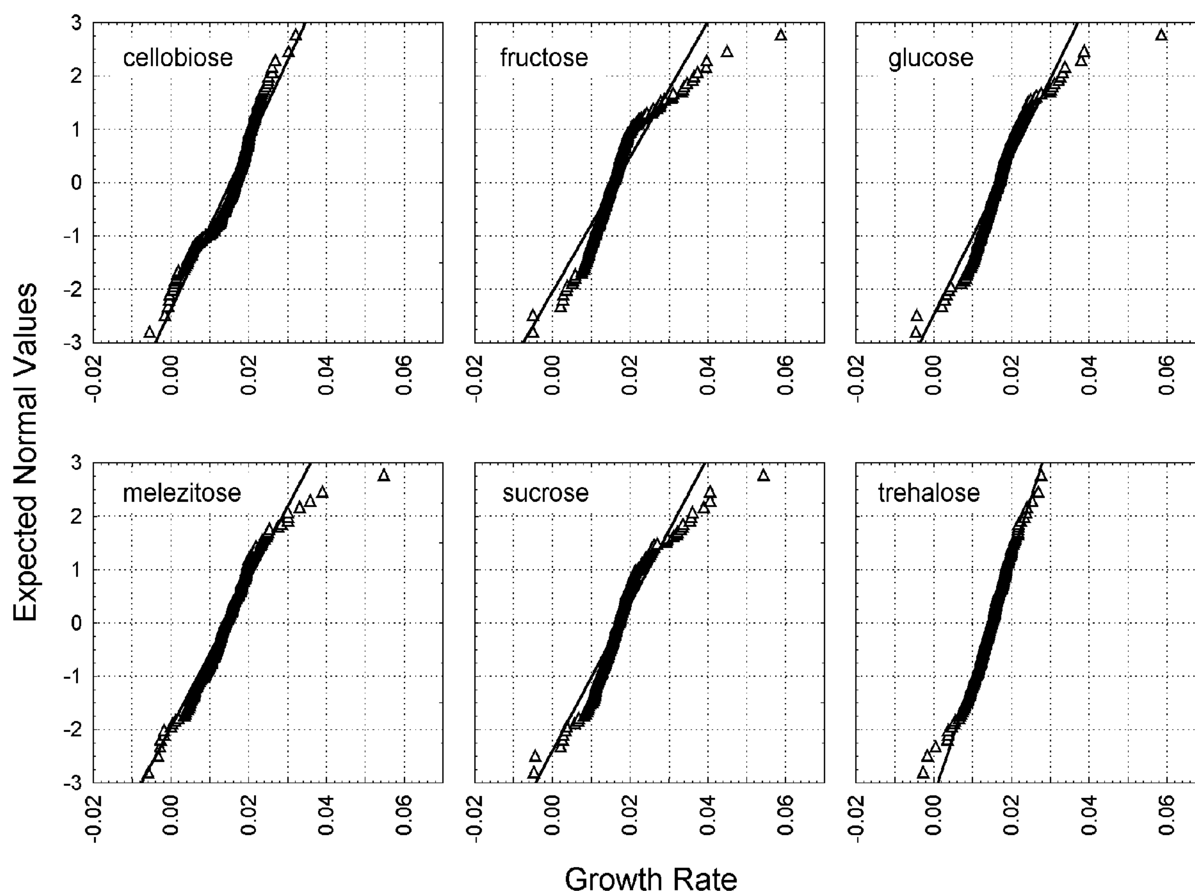

Fig. 5. Normal probability plots for growth rates of 250 yeast strains on six different sugars. The triangle symbols are the expected normal values of the rates plotted against the actual growth rates (as absorbance units per hour). The straight line drawn on each plot represents the expected values if the points were sampled from normal distribution. Deviation from the line at higher growth rates is indicative of positive skewing due to higher growth rates. The range of growth rate on each sugar is indicated by the spread along the growth rate axis.

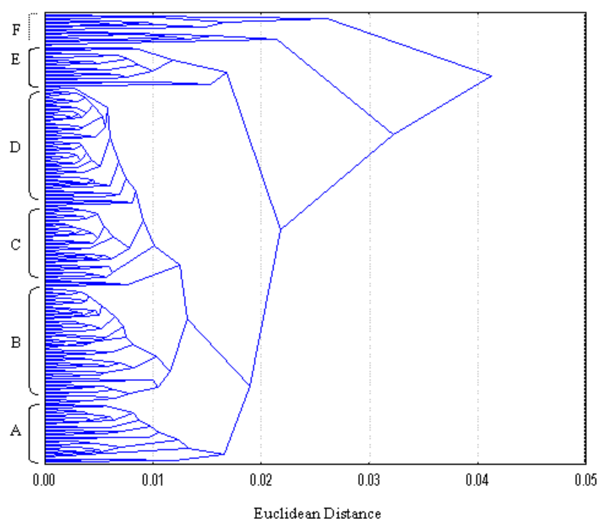

Fig. 6. Dendrogram of 250 yeast strains as characterized by growth rate on six different sugars. Cluster analysis was done using an unweighted pair-group average linkage method. Scale is based on Euclidean distance, and branches at lower Euclidean distances represent closer similarity. Yeast strains utilizing the same sugars and at similar rates are clustered together. Five major clusters are identified with the letters A through E. The sixth group, labeled F, is a collection of several small clusters.

($C_{12}H_{22}O_{11}$) or sucrose (both disaccharides) by the removal of a glucose monomer. These two disaccharides can be further hydrolyzed to glucose and fructose. It is probable that strains capable of hydrolyzing one or both linkages in the melezitose molecule would also utilize the resulting glucose and/or fructose monomers. These facts may explain the location of melezitose near the sucrose, glucose, and fructose cluster. Cellobiose, a b-1-4 disaccharide of glucose, was separate from other sugars and the location may reflect the occurrence and activity of the b-D-glucosidases in the yeast population under study.

Growth Rates as Influenced by Yeast Strain

When analyzed with normal probability plots, growth rates on cellobiose and trehalose were normally distributed, as indicated by the points falling on a straight line (Fig. 5). Growth rates on fructose, glucose, melezitose, and sucrose were approximately normally distributed but deviated from normal at the highest growth rates. The range of growth rates was smaller for cellobiose and trehalose compared with the other four sugars. The range of growth rates on the different sugars means that selection of bioremediation strains with higher rates of utilization should be feasible.

Cluster analyses of yeast strains based on patterns of sugar utilization revealed distinct grouping (Fig. 6). Five major clusters, labeled A through E, were identified and a sixth group, F, consisted of several small clusters. These clusters are best viewed as functional groupings and present a unique characterization of the yeast populations. In this analysis, yeast strains that utilized the same sugars at similar rates will group together. These groupings may be useful in selecting bioremediation agents, especially if future research shows a benefit of using a mixture of strains for bioremediation.

In an examination of 24 strains of yeast capable of utilizing more than one sugar, it was observed that utilization rates tended to be correlated (Fig. 7). Each bar in the figure represents a single strain and each segment within the bar represents the growth rate of that strain on an individual sugar. Strains represented by the tallest bars had high growth rates on many sugars. Strains represented by the shortest bars had low rates on many of the sugars. This indicates that selection of bioremediation agents with broad usage patterns and high rates of utilization on multiple sugars should be feasible.

CONCLUSIONS

All the sugars evaluated could be utilized by many of the strains tested. When assessed across all 250 strains, sucrose supported the highest average rate of growth, followed in order by glucose and fructose, melezitose, cellobiose, and trehalose. Since cellobiose utilization is often correlated with cellulase activity, cellobiose-utilizing strains should be evaluated for cellulase activity to avoid possible degradation of lint quality. Many strains could utilize more than one sugar and within the same strain, growth rates on the different sugars tended to be correlated. These results indicate that the naturally occurring yeast population in the San Joaquin Valley will be a suitable source for selecting bioremediation agents for whitefly and aphid honeydew. The variation in utilization rates found in the population indicates that selecting strains for rapid rates of utilization should be successful. Trehalulose, an important honeydew sugar, has yet to be tested, but

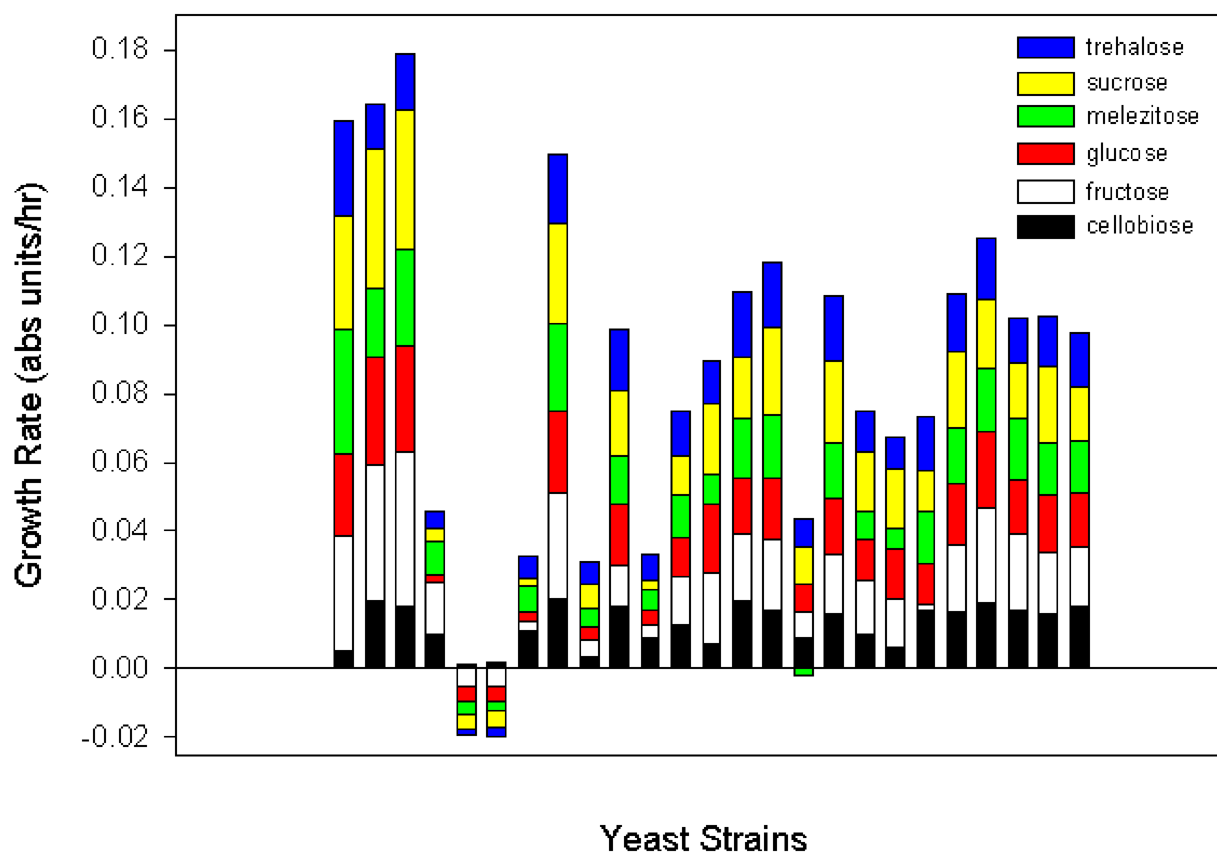

Fig. 7. Comparison of sugar utilization rates for 24 strains of yeast. Each bar represents a single strain, and bar segments represent the rate at which that strain utilized the indicated sugar. Growth rate is expressed as absorbance units per hour.

given its prevalence in whitefly honeydew, strains will probably be found that can degrade this sugar.

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