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Bio-ethanol Fermentation by the Bioconversion of Sugar from Dates by *Saccharomyces cerevisiae* Strains ASN-3 and HA-4

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Abstract: The essence of bioconversion of sugar containing materials by the use of screened microbial source is the significant need of today. *Saccharomyces cerevisiae* is one of the common and the cheapest source of bioconversion of substrate to the higher yield of bioethanol under the controlled optimization parameters. In the present study *Saccharomyces cerevisiae* strains ASN-3 and HA-4 were activated and finally used for ethanol fermentation from Dates. The results revealed that both strains are actively involved in fermentation process but it is concluded that strain HA-4 resulted higher cellular mass when inoculated in Malt Yeast extract Peptone Glucose medium supplemented with fructose and yeast nitrogen base and higher yield of ethanol was also observed when the activated strain were inoculated in dates syrup as substrate at 120 rpm, having pH 4.5 at 30°C at 600 nm after 72 h post incubation.

Key words: Ethanol fermentation, activated yeast, dates, optimization

Introduction

The dates are supposed to be a good diet for humans, which have a close touch with them. Date palms are widely spread in Mediterranian areas by Arabs and are cultivated globally (Farooqi, 1989; Pandey and Anita, 1990). Pakistan is fifth largest Date producing country in the world and District Khairpur is the largest area about 6076 square miles where more than 200 varieties are cultivated (Khusk, 1988; Murray, 1989). Chemically, ***Phoenix dactylefera*** L. variety Aseel consists of 75-80% glucose and fructose, fats tannin, vitamin and traces of minerals. This variety belongs to Arecaceae family, its parts like ashes of roots and leaves, base of spathe and flowers are useful in the daily life (Said ***et al.***, 1996; Qureshi ***et al.***, 2002).

Yeast is single-celled, most domesticated group of microorganisms belong to the kingdom Ascomycotina. They are ubiquitous in the natural world and their preferred niches in nature are on the surface of fruits and tree exudates and in dead and decaying vegetation, where they thrive on sugar material. They are of multiple economic, social and health significance and have been exploited unwittingly, since ancient times for the provision of food (leaved breed) e.g. *Saccharomyces cerevisiae*. This yeast is one of the oldest, exploited and best studied

microorganism in both old and new biotechnologies and is known to be the world's premier industrial microorganisms which readily convert sugars into alcohol and Co_2 in metabolic process called fermentation for example of alcoholic beverages viz. Beer, mead, cidar, sake and distilled sprits (Kurtzman and Fell, 1998; Walker, 1998).

People have been using natural products since the dawn of human history. A natural product is a product that is derived from plants, animals or microbial sources, primarily through physical processing, sometimes facilitated by simple chemical reactions such as acidification, basification, ion-exchange, hydrolysis and salt formation as well as microbial fermentations (Leung, 1980). Alcohol fermentation has traditionally been carried out in aqueous environment because of the ready solubility of reactant and product. Ethanol is infect a colorless solvent, volatile in nature, having spirituous smell, burning taste and also a source of consumable energy and a feedback. It is now synonym for energy. Its production by yeast from sugar crops is now a source of energy which is a dire need of today's economy (Moritz and Sheldon, 1996; Noor and Hameed, 1997; 1998; 1999b). In recent times, ethanol production for fuel use has been a subject of great interest and various researches are being carried out aiming at increasing or maximizing microbial ethanol production at cheaper cost (Bawa and Yoshiyuki, 1992).

Materials and Methods

The present study was conducted at the Microbiology laboratory and Biotechnology laboratory at Sindh University Jamshoro and the Microbiology research laboratory at Quaid-I-Azam University Islamabad. During this presentation fresh dates variety Aseel (***Phoenix dactyleifera*** L.) were purchased locally, their coast was peeled and later cut into smaller pieces. These pieces weighing 500 g were mixed with 500 ml of sterile distilled water to make 100% (w/v) date syrup. It was heated (not boiled) gently till the preparation of concentrated syrup, cooled, clarified and sugar concentration, moisture and ashes were determined according to Noor and Hameed (1999b), Miller (1959) and Noor and Hameed (1997), respectively.

Preparation of primary inoculum

Yeast, ***Saccharomyces cerevisiae*** strain ASN-3 and HA-4 were activated separately in 100 ml of Malt Yeast extract, peptone glucose (MYPG) broth (pH 4.5) in 400 ml Erlenmeyer flasks at various temperatures (28-36°C) and different agitation speeds (100-140 rpm). Absorption (Optical Density) was determined after every 24 h till 96 h at 600 nm Noor ***et al.*** (2001).

Preparation of secondary inoculum

One ml of primary inoculum (72 h old) was inoculated in 2 separate flasks of MYPG broth supplemented with 0.5 and 01% of fructose, respectively. This mixture was allowed at 120 rpm, pH 4.5, 30°C, 72 h incubation and O.D. was determined at 600 nm.

Preparation of tertiary inoculum

One ml of primary and secondary cultures of test strains were inoculated separately in 100 ml of yeast nitrogen base (YNB, pH 4.5) in 400 ml Erlenmeyer flasks with the concentration of 0.5 and 01%, respectively. These flasks were allowed at 120 rpm, 30°C, 72 h incubation and O.D. was determined at 600 nm.

Preparation of starter culture

One ml of 72 h old cultures of years strains ASN-3 and HA-4 were inoculated separately in 100 ml (in 400 ml Erlenmeyer flasks) of the fermentation medium composed of Malt and Yeast extract 0.3%, peptone from soy bean meal 0.5%, glucose 01% and fructose 01% and YNB 0.5% weight by volume, pH was adjusted at 4.5. These flasks were allowed for incubation for 72 h at 120 rpm, 30°C and the O.D. was determined at 600 nm. Growth rate (K) and total cell dry weight were also determined after every 24 h.

One ml of starter cultures were inoculated in 99 ml of clarified date syrup to make 1% v/v mixtures of substrate and inocula under hygienic conditions and allowed at 120 rpm, pH 4.5, 30°C for 72 h incubation in orbital shaker incubator. Later the fermented medium was allowed for fractional distillation at 76°C for ethanolic yield. Distilled ethanol was estimated by potassium di-chromate method of Chodhary ***et al.*** (1986).

Results

The results in Tables 1-5 show the different optimization parameters including various temperatures (28-30°C) and agitation speeds (100-140 rpm) in orbital shaking incubator for activation of yeast strains ASN-3 and HA-4 screen out an efficient strain to be used as final starter culture in M.Y.P.G. broth having pH 4.5 at 600 nm. After every 24 h till 96 h incubation.Tables 1-3

Table 1: Determination of maximum growth (O.D.) Of *S. **Verevisiae*** strains ASN-3 and HA-4 in M.Y.P.G. broth in various temperatures (28-36 °C) at 100 rpm. agitation speed, A_{600} after 96 h post incubation

Incubation period (h)	Strain	Various temperatures (°C) 28	30	32	34	36
24	ASN-3	0.02	0.04	0.03	0.03	0.01
	HA-4	0.04	0.06	0.06	0.04	-0.02
48	ASN-3	0.04	0.07	0.05	0.04	0.02
	HA-4	0.06	0.10	0.06	0.05	0.03
72	ASN-3	0.10	0.12	0.07	0.06	0.04
	HA-4	0.13	0.15	0.10	0.07	0.05
96	ASN-3	0.07	0.09	0.05	0.02	0.01
	HA-4	0.04	0.06	0.07	0.04	0.01

Table 2: Determination of maximum growth (O.D.) of *S. cerevisiae* strains ASN-3 and HA-4 in M.Y.P.G. broth on various temperatures (28-36°C) at 110 rpm agitation speed, A_{600} after 96 h post incubation

Incubation period (h)	Strain	Various temperatures (°C) 28	30	32	34	36
24	ASN-3	0.04	0.07	0.06	0.04	0.02
	HA-4	0.07	0.13	0.10	0.07	0.05
48	ASN-3	0.06	0.10	0.08	0.06	0.03
	HA-4	0.10	0.16	0.13	0.09	0.05
72	ASN-3	0.09	0.16	0.12	0.10	0.06
	HA-4	0.13	0.21	0.18	0.13	0.09
96	ASN-3	0.06	0.12	0.09	0.06	0.03
	HA-4	0.10	0.15	0.15	0.10	0.00

Table 3: Determination of maximum growth (O.D.) of *S. Cerevisiae* strains ASN-3 and HA-4 in M.Y.P.G. broth on various temperatures (28-36°C) at 120 rpm agitation speed, A_{600} after 96 h post incubation

Incubation period (h)	Strain	Various temperatures (°C) 28	30	32	34	36
24	ASN-3	0.12	0.21	0.18	0.14	0.10
	HA-4	0.18	0.29	0.26	0.22	0.15
48	ASN-3	0.14	0.27	0.23	0.17	0.12
	HA-4	0.22	0.35	0.31	0.24	0.18
72	ASN-3	0.18	0.34	0.27	0.21	0.15
	HA-4	0.34	0.42	0.28	0.27	0.20
96	ASN-3	0.16	0.30	0.20	0.21	0.11
	HA-4	0.29	0.38	0.23	0.20	0.17

indicate the gradual increase of yeast cell mass (0.34 and 0.42) at 100-120 rpm, 30°C and 600 rpm. After ward the cell mass decreases (Table 4, 5).

Tables (6, 7) shows the higher O.D. of test strain ASN-3 (0.55, 0.64), HA-4 (0.60, 0.72) and (0.59, 0.51), (0.70, 0.63) when grown in M.Y.P.G. broth supplemented with 1% fructose and 0.5% YNB, respectively. In the same medium growth rate (K) and total cell dry weight were also determined after every 24 h till 96 h incubation which resulted 0.0197, 0.032 and 0.0199, 0.042 (Table 6) and

Table 4: Determination of maximum growth (O.D.) of *S. Cerevisiae* strains ASN-3 and HA-4 in M.Y.P.G. broth on various temperatures (28-36°C) at 130 rpm agitation speed, A_{600} after 96 h post incubation

Incubation period (h)	Strain	Various temperatures (°C) 28	30	32	34	36
24	ASN-3	0.10	0.16	0.15	0.13	0.10
	HA-4	0.17	0.20	0.19	0.15	0.12
48	ASN-3	0.14	0.22	0.20	0.15	0.11
	HA-4	0.25	0.28	0.24	0.18	0.14
72	ASN-3	0.16	0.29	0.24	0.17	0.13
	HA-4	0.31	0.36	0.29	0.23	0.18
96	ASN-3	0.13	0.25	0.21	0.14	0.09
	HA-4	0.27	0.32	0.24	0.18	0.13

Table 5: Determination of maximum growth (O.D.) of *S. Cerevisiae* strains ASN-3 and HA-4 in M.Y.P.G. broth on various temperatures (28-36°C) at 140 rpm agitation speed, A_{600} after 96 h post incubation

Incubation period (h)	Strain	Various temperatures (°C) 28	30	32	34	36
24	ASN-3	0.12	0.15	0.13	0.10	0.08
	HA-4	0.12	0.16	0.16	0.13	0.10
48	ASN-3	0.14	0.17	0.15	0.11	0.09
	HA-4	0.15	0.20	0.18	0.14	0.11
72	ASN-3	0.19	0.20	0.18	0.14	0.10
	HA-4	0.19	0.25	0.21	0.16	0.12
96	ASN-3	0.16	0.18	0.16	0.12	0.07
	HA-4	0.17	0.23	0.19	0.10	0.09

0.0199, 0.038 and 0.0205, 0.046 growth rate and total cell dry weight of strains ASN-3 and HA-4, respectively. Fractional distillation of ferments date syrup at 76°C resulted 0.73 and 0.81% of ethanolic yield by strains ASN-3 and HA-4, respectively.

Table 6: Determination of optimization parameters (O.D. at various temperatures, growth rate and total cell dry weight) of test strains in M.Y.P.G. broth supplemented with 0.5 and 01% fructose at 120 rpm, pH 4.5, A_{600}, after every 24 h incubation

	Incubation period (h)							
	24		48		72		96	
	ASN-3	HA-4	ASN-3	HA-4	ASN-3	HA-4	ASN-3	HA-4
O.D. at various temperatures (°C)								
28	0.40	0.45	0.43	0.49	0.48	0.54	0.45	0.51
	0.46	0.55	0.50	0.59	0.54	0.64	0.50	0.60
30	0.44	0.51	0.47	0.51	0.55	0.60	0.48	0.57
	0.50	0.62	0.53	0.60	0.65	0.72	0.55	0.68
32	0.41	0.48	0.44	0.51	0.48	0.55	0.45	0.50
	0.50	0.56	0.52	0.61	0.55	0.66	0.53	0.62
34	0.37	0.45	0.39	0.48	0.42	0.51	0.38	0.48
	0.46	0.52	0.48	0.55	0.50	0.59	0.47	0.57
36	0.32	0.40	0.34	0.42	0.38	0.45	0.33	0.41
	0.41	0.47	0.42	0.49	0.45	0.51	0.39	0.46
Growth rate (K)	0.0177	0.0188	0.0188	0.0194	0.0197	0.0199	0.0179	0.0185
Total cell dry weight	0.019	0.025	0.028	0.032	0.038	0.042	0.034	0.030

Fructose concentration percent (0.5, 01)

Discussion

Dates, variety Aseel (***Phoenix dactylefera*** L.) are the wind pollinated diocious plants that give fruits till hundred years and posses the nutritions substances like vitamins of B-complex (B1 and B2), water soluble vitamins (A, C, D), traces of minerals mainly Ca, Mg, Na, Cu, S, P and little concentration of Zn. Dates are also considered as an additional source of protein with quantities of essential amino acids (Table 8) in addition to their known sugar content (Pandey and Anita, 1994; Said ***et al.***, 1996; Qureshi ***et al.***, 2002; Ismaili ***et al.***, 2002).

Yeast shows a marvelous and harsh response in its oxidative metabolism region due to the spontaneous cell synchronization when cultivated in different laboratory media. Several workers changed the nutritional substances in media in order to enhance the growth/cell mass of ***Saccharomyces cerevisiae*** but the activation according to Noor ***et al.*** (2001) in malt medium (rich in maltose/α-glucoside) provides a marvelous nutritional requirement which supports the higher cellular mass and enzymatic activity. It is due to the utilization of sugars in the medium which

Table 7: Determination of optimization parameters (O.D. at various temperatures, growth rate and total cell dry weight) of test strains in M.Y.P.G. broth supplemented with 0.5 and 01% YNB at 120 rpm, pH 4.5, A_{600}, after every 24 h incubation

	Incubation period (h)							
	24		48		72		96	
	ASN-3	HA-4	ASN-3	HA-4	ASN-3	HA-4	ASN-3	HA-4
O.D. at various temperatures (°C)								
28	0.46	0.55	0.50	0.58	0.55	0.63	0.51	0.58
	0.37	0.46	0.41	0.50	0.48	0.57	0.43	0.52
30	0.53	0.60	0.56	0.64	0.59	0.70	0.51	0.62
	0.42	0.50	0.46	0.54	0.51	0.63	0.48	0.60
32	0.48	0.56	0.53	0.60	0.55	0.66	0.52	0.61
	0.42	0.55	0.43	0.58	0.47	0.62	0.44	0.60
34	0.35	0.53	0.47	0.55	0.50	0.59	0.46	0.56
	0.37	0.50	0.39	0.52	0.42	0.55	0.42	0.51
36	0.31	0.47	0.33	0.50	0.36	0.52	0.34	0.48
	0.33	0.42	0.36	0.45	0.40	0.49	0.37	0.44
Growth rate (K)	0.0181	0.0190	0.0187	0.0197	0.0199	0.0205	0.0193	0.0199
Total cell dry weight	0.028	0.037	0.032	0.040	0.038	0.046	0.034	0.040

YNB concentration percent (0.5, 01)

is based on the homologous and unlinked *MAL* loci, which encode maltase permease, maltose and a positive regulatory protein by active transport across the plasma membrane and subsequently its hydrolysis by cytoplasm glucosidases (Boris and Pedro, 2001; Claudia and Boris, 2001).

Glucose is a monosaccharide sugar and is generally used in media to provide carbon and energy source to all living cells. It was supplemented in malt medium for the increased growth. It also supports the yeast to grow on non-fermentable carbon source causing induction of various signal transduction pathways. During cultivation practices of yeast growth fructose was also added to provide multiple sugar source along with glucose for the rapid enzymatic activity in the medium under the harsh environmental conditions. Instead of other nitrogen sources the peptone from soybean meal and yeast nitrogen base was supplemented. Multiple sugar source along with the nitrogen source supported the enhanced enzymatic activity due to the adaptation of new enzymes during the lag phase, which stimulate gluconeogenesis and the mitochondrial activity (Michelle ***et al.***, 1998) which result in the high ethanol recovery when the

Table 8: Amino acid content (dry basis) of Dates (***Phoenix dactylefera*** L.) Variety Aseel of Khairpur district

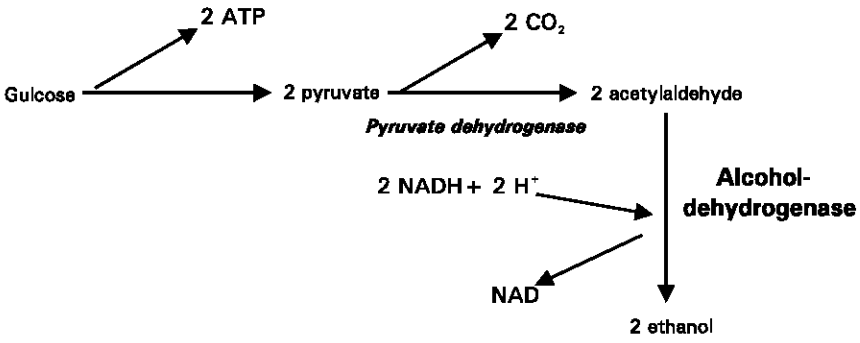
Amino acids	mg 100 g^{-1}	Amino acids	mg 100 g^{-1}
Lysine	104	Proline	205
Histidine	40	Glycine	205
Arginine	115	Alanine	125
Aspartic acid	272	Valine	98
Threonine	77	Methionine	45
Serine	102	Isoleucine	40
Glutamic acid	410	Leucine	110

test strain is inoculated in Date syrup which is considered as a good protein food but low in nitrogen (calorie 0.63) content (Ismaili ***et al.***, 2002). Yeast cells also require macro nutrients viz. sources of C, N, O, S, P, P and Mg at the millimolar level, trace elements at the micromolar level and other growth factors including the purines, pyrimidines, nucleotides and nuclerosides, amino acids, fatty acids, sterols, vitamins and other miscellaneous compounds e.g. polyamines and choline which serve the vital functions as components of co-enzyme.

Saccharomyces cerevisiae are aerobic microorganisms, their multiplication and cellular biomass relies on respiratory, rather than fermentative metabolism. Thus, yeast is propagated under fully aerobic conditions and the rate of sugar feeding is carefully controlled to prevent the cells from converting sugar to ethanol in the growth medium. In this way, respiration predominates, energy generation in the form of ATP is enhanced and the production of yeast biomass is enhanced (Walker, 2000).

During bioconversion of date syrup several micro elements and trace elements such as, Mg, Ca, K and Zn improve the structural and enzymatic regulatory activities during growth and metabolism, enhance the osmoregulation and promote the activity of other metalloenzymes like cysteine de-sulfydrase, stimulates the uptake of maltose in cells, activates riboflavin synthesis and also increases protein content in the fermentation yeast, respectively (Michelle ***et al.***, 1998).

Yeat, as fermentative microorganisms are able to use organic substrates anaerobically as electron donor, electron acceptor and carbon sources. During alcoholic fermentation of sugars,



S. cerevisiae reoxidize the reduced co-enzyme NADH to NAD in terminal step reactions from pyruvate which is needed for continued sugar breakdown during glycolysis and to maintain the redox balance in the cells as shown in reaction.

References

Bawa Demuyakor and Yoshiyuki Ohta, 1992. Promotive action of ceramics on yeast ethanol production, and its relationship to pH, glycerol and alcohol dehydrogenase activity. Appl. Microbial Biotehnol., 36: 717-721.

Boris, U., Stambuk and P.S.de Araujo, 2001. Kinetics of active α-glucoside transport in *Saccharomyces cerevisiae*. FEMS Yeast Res., 1: 73-78.

Chodhary, M.Y., I. Mahmood, M.A. Shah, 1986. Ethanol production in batch and fed-batch systems by yeast. Pak. J. Biochem., 19: 1-3.

Claudia, H. and B.U. Stambuk, 2001. Calorimetric determination of active α-glucoside transport in *S.cerevisiae*. J. Microbiol. Methods. 46: 253-259.

Farooqi, I., 1989. Nibatat-e-Quran. Sheikh Muhammad Bashir and Sons, Urdu Bazar, Lahore, pp: 23-36.

Ismaili N.J., S.A. Qayoom and M.M. Saleh, 2001. Amino acid composition of three varieties of Date, in Khairpur, Sindh, Pakistan, Scientific Khyber, 15: 65-68.

Khusk, M.K., 1998. Role of inorganic constituents in development of dates fruit in Khairpur. Ph.D. Thesis, University of Sindh Jamshoro, Pakistan, pp: 11-13.

Kurtzman, C.P. and J.W. Fell, 1998. The Yeast: A taxonomic study. Elsevier Science Publication, pp: 37-48.

Leung, A.Y., 1980. Encyclopedia of Common Natural Ingredients, 1st ed, John Wiley and Sons Inc., pp: 1.

Mortiz, J.W. and J.B.D. Sheldon, 1996. Simultaneous saccharification and extractive fermentation of cellulose substrates. Biotechnol. Bioeng., 49: 504-511.

Miller, G.L., 1959. Use of di-nitrosalisylic acid (DNS) for determination of reducing sugars. Annal. Chem., 31: 426-428.

Michelle M.C.M., J.B. Arie J. Verkleji and C.T. Verrips, 1998. Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than glucose flux. J. Biol. Chem., 273: 24102-24107.

Noor, A.A. and A.Hameed, 1997. Yeast bioconversion of glucose from molasses to ethanol by *Saccharomyces cerevisiae*. Pak. J. Biochem. and Mol. Biol., 30: 97-103.

Noor, A.A. and A. Hameed, 1998. A multi step optimization study for maximum growth of saccharophilic yeast *Saccharomyces cerevisiae* strain ASN-3: A three-in-One approach. Sci. Sindh, Pakistan, 5: 1-8.

Noor, A.A. and A. Hameed, 1999. Optimization studies for bioconversion of corn steep liquor to ethanol by *Saccharomyces cerevisiae* strain KA-1 and AAN-2. Pak. J. Biol. Sci., 2: 137-140.

Noor, A.A., G.A. Maka, M.A. Mahar, K. Bhatti and A. Hameed, 2001. Baker's yeast strain HA-4 and the growth parameters. Pak. J. Biol. Sci., 4: 569-571.

Pandey, B.P. and Anita, 1990. Economic Botany, S.Chand and Co. Ltd., Ram Nagar, New Delhi-110055, India, pp: 7-99.

Qureshi, R., G.R. Bhatti and G.S. Jakhar, 2002. Taxonomy and Ethanobotany of Date Palm in District Khairpur. Accepted in Hamdard Medicus, July-Sept., 2002.

Said, H.M., M.A. Barkati and K.K. Ahmed, 1996. Mufeed Ghizain, Dawain. Baital-Hikamat, Muhammad Bin Qasim ave., Madinat-al-Hikmah Karachi, Pakistan, pp: 105-106, 171-172.

Walker, G.M., 1998. Yeast Physiology and Biotechnology, 2nd ed, Jhon Willey and Sons UK, pp: 11-17.

Walker, G.M., 2000. Encyclopedia of Microbiology, Academic Press, 4: 939-953.