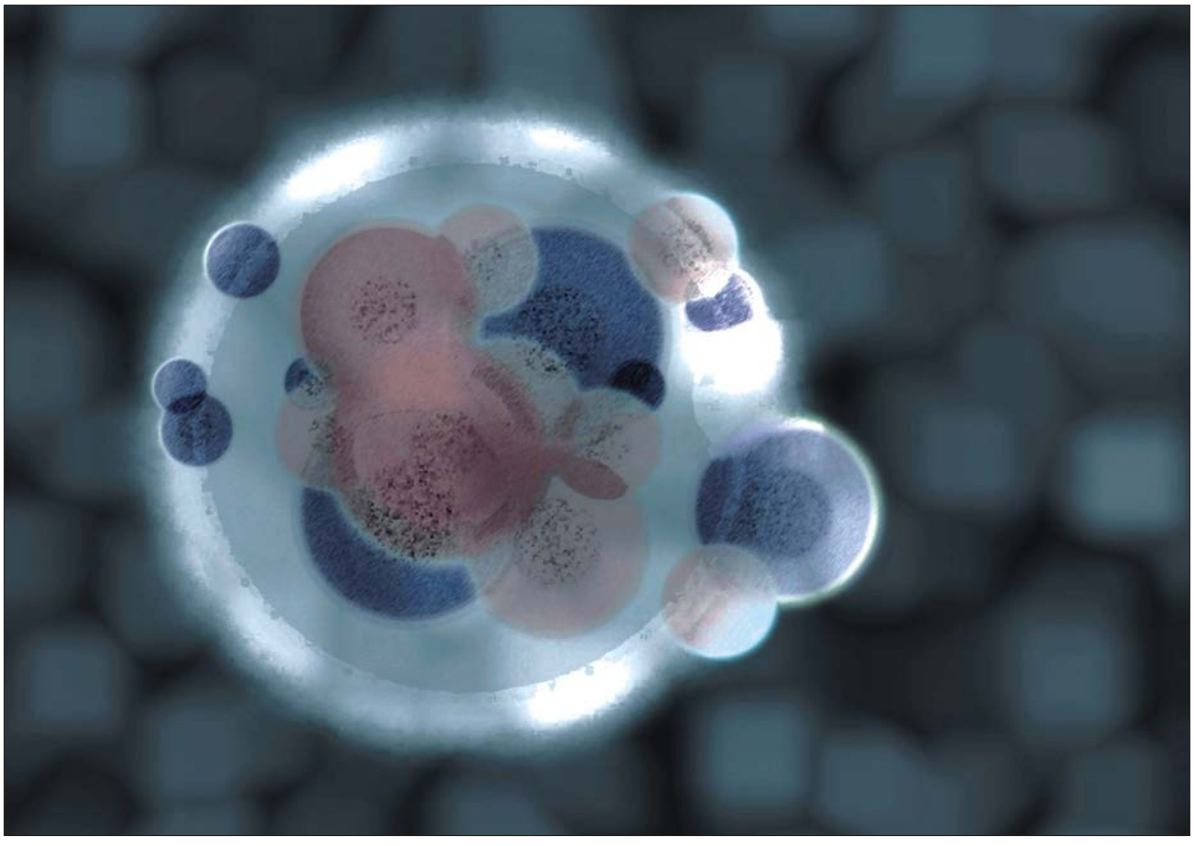

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Bioconversion of Raw Starch to SCP by Coculture of *Cryptococcus aerius* and *Saccharomyces cerevisiae*

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Abstract: Simultaneous degradation and conversion of unhydrolysed wheat starch to high protein content SCP by monoculture of an isolated amylolytic yeast, *Cryptococcus aerius* and co-culture of *Cryptococcus aerius* and *Saccharomyces cerevisiae* was investigated. Amylolytic activity, starch degradation rate, biomass and protein content of biomass increased several fold in coculture versus the monoculture due to the synergistic metabolic interactions between the two species. Under optimal condition, in monoculture of *C. aerius* 10.02 g L^{-1} biomass produced after about 78 h with 27.8% protein content and >84% efficiency; while with optimal ratio of *C. aerius* to *S.cerevisiae* (5% v/v:5% v/v) the cultivation was improved totally. In coculture, total cultivation time decreased to 58 h with about 94% efficiency in biomass production, while biomass and total cellular crude protein of biomass increased from 10.02 to 11.22 g L^{-1} and 27 to 44.2%, respectively. These results indicate that simultaneous degradation of raw starch to SCP can be conducted efficiently by using coculture of the amylolytic yeast, *C. aerius* and a nonamylolytic yeast, *S. cerevisiae*.

Key words: Biomass, wheat, amylase, SCP, protein

INTRODUCTION

Starch is the most important storage polysaccharide in plant cells, it is abundant in tubers and seeds especially corn, starch is a polymer of glucose residue[1]. Large volumes of starchy feedstock such as corn, represent an important biomass resource for production of microbial biomass (SCP), gaseous or liquid fuels and chemicals; because the chemical composition and high density of starch, compared to other forms of biomass, facilitates prolonged storage and decrease transportation and pretreatment costs[2,3]. The rapid growth rate and high protein content of microbes and their ability to utilize inexpensive feedstock as sources of carbon and energy for growth have made microorganisms prime candidates for use as human food and animal feed protein supplements[4]. General factors have limited the utilization of microbial proteins in human nutrition, such as toxicological barriers, nutritive value and functional properties but significantly less nutritive problems are being concerned with the application of SCP in animal nutrition[5]. The well known yeasts do not secrete extracellular amylolytic enzymes and are therefore unable to utilize starch for the production of microbial biomass[6-8]. Initial studies aimed at the elimination of the enzymatic liquefaction and saccharification step by symbiotic coculture of amylolytic and sugar-fermenting organisms have been promising, for example, in the Symba process for single cell protein production from potato processing waste, the enzymatic liquefaction and saccharification process was eliminated by using a coculture of *Endomycopsis fibuligera* (amylolytic yeast) and *candida utilis* (nonamylolytic sugar utilizer)[5]. Direct conversion of (manioc) starch to ethanol; by *Saccharomyces diastaticus* has been suggested; however, prior treatment of the starch with α-amylase was required for obtaining efficient fermentation of starch[9,10]. In Iran, the production of corn starch has increased in recent years, due to increase in wheat and potato production and their related processing technology. According to guidelines for waste management in Iran, this kind of waste must refine or improve; so the purpose of this study was to develop and evaluate a simultaneous single step system for the enhance fermentation of corn starch to SCP by symbiotic coculture of an amylase producing yeast species *Cryptococcus aerius* and *Saccharmomyces cerevisiae*.

MATERIALS AND METHODS

Microorganism: Amylase-producing yeast was isolated from corn starch processing industrial wastes after

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enrichment in growth media plus 0.1 g L^{-1} of chloramphenicol as bacteriocide antibiotic. The ability of the yeast to hydrolyze starch was tested though starch assimilation test on solid growth medium 15 g L^{-1} agar by the axuonographic method[11,12].

S. cerevisiae was obtained from the Persian type Culture Collection (PTCC). Yeasts were maintained on yeast extract glucose chloramphenicol agar (GYC) at 4°C.

Media: The growth medium contained, in gram per liter: Soluble starch, 10; ammonium sulphate, 0.5; K_2HPO_4, 0.5; NaH_2PO_4, 0.14; magnesium sulphate, $7H_2O$, 0.2; calcium chloride 1 H_2O, 0.1; yeast extract 1. The growth medium was used for *C. aerius* inocula preparation and enzymatic assay.

The main medium for SCP production from starch was identical to the growth medium except that the raw wheat starch (30 g L^{-1}) substituted. The desired initial pH (5.5) was obtained by addition of HCl (1 N) and NaOH (1 N). Tap water was used for all medium preparation.

Preparation of inocula: *C. aerius* inocula were prepared by inoculating slant cultures to 100 mL of sterile growth medium contained in 500 mL Erlenmeyer flasks. The flasks were incubated with shaking (200 rpm) at 30°C for 36 h. *S. cerevisiae* inoculum was prepared in the same way as the amylolytic yeast inoculum except that malt extract broth (pH 5.5) was used (instead of growth medium).

Enzyme assay: Three replicate flask (500 mL) contained 140 mL growth media (with 5 g L^{-1} soluble starch) were inoculated with 10 mL of inocula and maintained in the same condition described for inocula preparation. At regular time intervals, samples were taken and total extracellular amylase activity was tested in its supernatant. Total amylolytic activity was determined by measuring reducing sugar released from soluble starch[1] in which the reduction of 3,5-dinitrosalicylic acid (DNS) by reducing sugars was determined[13]. A standard curve for this colorimetric assay was constructed, using glucose as the standard [1]. One unit of amylolytic activity (U) was defined as the amount of enzyme in 1 mL that liberates 1 μmol of reducing sugar from soluble starch in 1 min at 30°C.

SCP production procedure: SCP production was carried out in 500 mL Erlenmeyer flasks containing 150 mL of the main medium in batch mode condition. The flasks were sterilized by autoclaving at 121°C for 15 min. Grown *S. cerevisiae* centrifuged at 4500 rpm for 10 min and cells transferred into main media as inocula. Various amounts of grown *C. aerius* were centrifuged at 4500 rpm for 10 min and used as inocula. The flasks incubated at 30°C with shaking (200 rpm). Total cultivation times differed according to the time needed for reducing sugar depletion.

Efficiency of biomass production on raw wheat starch was estimated by using formula Keer[1]: glucose (100 m L^{-1}) x 0.93 = starch (100 m L^{-1}) and comparing the biomass produced on 30 g L^{-1} of raw starch and 32.2 g L^{-1} of pure glucose.

Analytical procedure: Unless otherwise mentioned, samples (10 mL) were collected from a given flask and centrifuged at 4500 rpm for 10 min to remove cells and supernatant fluid was used for determining residual starch concentration, glucose concentration, Amylase activity and the pellet was used for biomass determination.

Residual starch determination: An iodine reagent was prepared freshly by diluting 1 mL of stock solution (0.5% I_2, in 5.0% KI) into 500 mL of distilled water containing 5 mL of 5 N HCl. For the measurement, 5 mL of the iodine reagent was mixed with 0.2 mL of starch solution (With more than 1.0% starch solution, the starch solution was diluted ten times with distilled water) then the temperature was adjusted at 30°C in bathwater for 3-4 min and the absorbance was measured at 620 nm against a blank (0.2 mL of water in 5 mL of iodine reagent)[14].

Reducing sugar determination: Free glucose was measured by enzymatic method (Glucose oxidase). The supernatant sample was boiled to denature all enzymes for 10 min, after that it was used for glucose determination. Reducing sugar was determined by 3, 5-Dinitrosalicylic acid (DNS)[13].

Cell biomass determination: Samples were centrifuged in preweighed tubes for 10 min at 4500 rpm, washed twice (9 g L^{-1} NaCl), dried for 24 h at 110°C and stored in a desiccator before weighing.

Total cellular crude protein determination: In the end of cultivation time, 5 mL of fermentation broth was centrifuged (4500 rpm, 10 min), the pellet was washed twice (5 mL cold 9 g L^{-1} NaCl). Then 3 mL of 1 M NaOH was added to the pellet and it was boiled for 10 min[15]. After cooling, the protein content was measured by Biuret method. Bovin serum albumin was used as standard[16].

Statistical analyses: Mean value of at least three separated experiments in identical flasks has been presented.

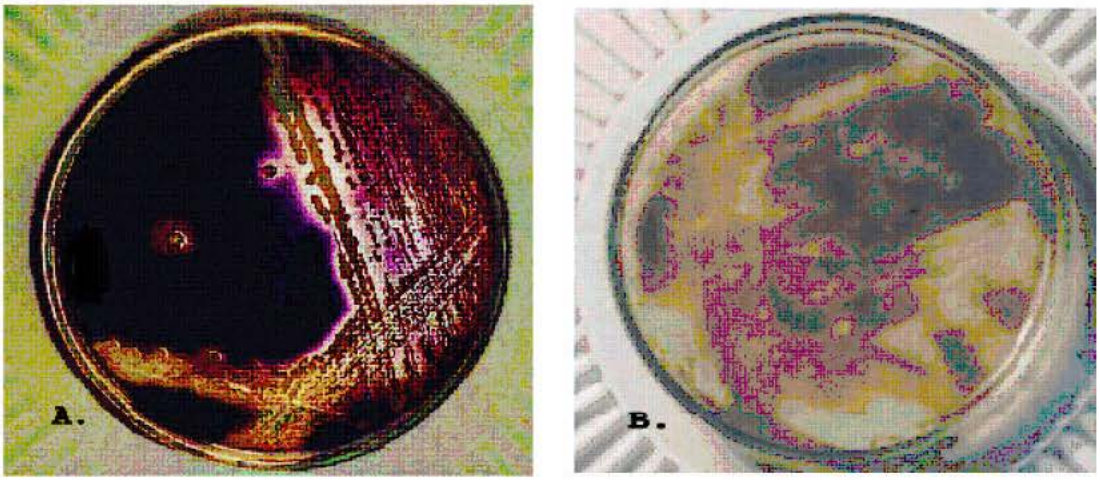

Fig. 1: Soluble (A) and raw (B) starch hydrolyzing by *C. aerius* in growth medium at 30°C in 24 h

Table 1: Some biochemical tests for two yeast strains

Reactions\ Microorganisms		*C. aerius*	*S. cerevisiae*
Fermentation	Glucose	Neg	Pos
	Galactose	Neg	Neg
	Sucrose	Neg	Pos
	Maltose	Neg	Pos
	Lactose	Neg	Neg
	Trehalose	Neg	Pos
Assimilation reactions	Glucose	Pos	Pos
	Galactose	Pos	Neg
	Sucrose	Pos	Pos
	Maltose	Pos	Pos
	Lactose	Pos	Neg
	Trehalose	Pos	Pos
	Raffinose	Pos	Neg
	Melibiose	Pos	Neg
	Soluble starch	Pos	Neg
	D-Xylose	Pos	ND
	L-Arabinose	Pos	ND
	D-Arabinose	Pos	ND
	D-Ribose	Pos	ND
	Ethanol	Pos	Pos
	D-Manitol	Pos	ND
	Citrate	Pos	ND
	Inositol	Pos	ND
	Vitamin -free	Growth	No growth
Nitrogen assimilation	Ammonium	Pos	Pos
	Urea	Pos	Pos
	Nitrate	Pos	Neg
	Nitrite	Pos	Neg
Growth at	19°C	Pos	ND
	25°C	Pos	Pos
	34°C	Pos	Pos
	40°C	Neg	ND

ND: Not Determined, Pos: Growth, Neg:: No Growth

RESULTS AND DISCUSSION

Isolation and identification of amylase producing yeast: The starch hydrolyzing yeast which was isolated from corn starch processing industrial waste was identified by observation of morphological characteristics and by physiological tests such as fermentation, carbon assimilation, nitrogen assimilation, urease test, Diazonium blue B color test, etc. The isolated amylase producing yeast was an obligate aerobic yeast, that only assimilated sugar such as glucose, galactose, sucrose, maltose and starch. It wasn't able to ferment any sugar; it means that under anaerobic condition with 20 g L^{-1} of sugar it can not produce gas or acid[17]. It could assimilate nitrate and nitrite as nitrogen source and had a growth temperature range between 20-35°C. It could grow in vitamin free media; growth in vitamin free media is economically desirable for industrial application. Based on the above characteristics and the others (Table 1) the isolated yeast was identified as *Cryptococcus areius*. It had no pathogenic effect[17]. After isolation and identification it was tested for its potential to produce amylases on solid medium with soluble and raw wheat starch as carbon source. *C. aerius* showed a wide and clear hydrolysis halo on the growth medium (Fig. 1). Comparing the results of this study with pervious studies, shows that various specious of *Cryptococcus* when grown on starch-containing medium, exhibits high amylase production after 24 h of cultivation[18,19],on the other hand large digesting halo formed when *C. aerius* was grown on raw wheat starch medium(Fig. 1B), so this results indicated that this yeast might secret some amylases that could digest raw starch; as previous study mentioned[19].

Amylase production by *C. aerius*: Preliminary experiments using various initial pH values (2-9) and different cultivation temperature had shown that amylase

production by *C. aerius* was highest in a medium with initial pH between 4.5-6 at 30°C, therefore we selected 5.5 as the initial pH and 30°C as the optimum temperature for further investigation. In a another preliminary experiment the effect of starch concentration on starch degradation rate was tested, since it would be more desirable for industrial application to be able to utilize high substrate concentration, 10-70 g L^{-1} soluble starch was used in mono culture of *C. aerius*, the results showed that when starch concentration increased from 30 g L^{-1}, the starch degradation rate decreased rapidly; since *C. aerius* is an obligate aerobic species and starch gelatinization after heat treatment increases, so probably by increasing in starch concentration, the oxygen transfer rate decreases and as the result starch degradation rate, so 30 g L^{-1} starch concentration was selected for further experiments.

A typical pattern for the time course of starch degradation and amylase production (Fig. 2 and 3) showed that starch hydrolysis proceeded quickly during the early exponential phase but low level of reducing sugars were detected. The maximum amount of extracellular amylase was produced at the end of exponential phase and the pH was lowered to about 4.0. As ammonium sulphate was the basis of nitrogen source for *C. aerius*, probably by using NH_4^+ leaving SO_4^{2-} in medium causes a decrease in pH. As previously reported for several yeast species, amylase secretion occurs at the end of exponential growth phase[20,21]. Starch degraded in the early stage of cultivation and a rapid loss of iodine staining was found but at this time there was a low level of extracellular amylase in media (Fig. 2); this probably reflects the involvement of cell-bound activity in early starch hydrolysis. Glucose production proceeded after the starch degradation and simultaneously by assimilation of reducing sugar, biomass increased rapidly, it was demonstrated that probably there was a negative effect of reducing sugar (e.g. glucose) on amylase production and while glucose increased in media amylase secretion decreased. According to pervious studies on amylase secretion patterns by various yeasts, three different patterns have been described for extracellular amylase production[22]; a rapid loss of iodine-staining combined with a high final level of reducing sugar probably indicated the presence of both α-amylase and glucoamylase for *C. aerius*.

Effect of glucose on starch degradation by *C. aerius*: Glucose probably had inhibitory effect on amylase production, so the objective of first series of experiments was to test the hypothesis that if glucose could repress starch degradation by *C. aerius*. The effect of starch and glucose concentration on amylase activity has been

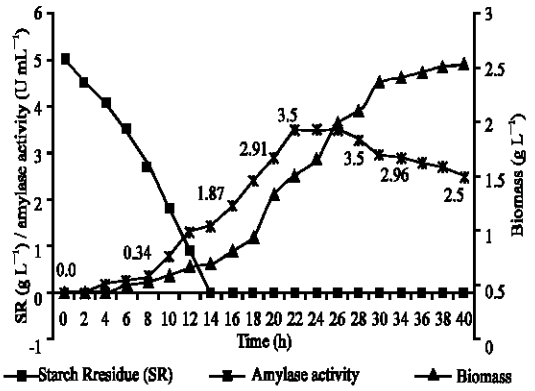

Fig. 2: Time course of starch degradation, amylase secretion and biomass production by *C. aerius* growing in starch medium (5 g L^{-1}) at 30°C

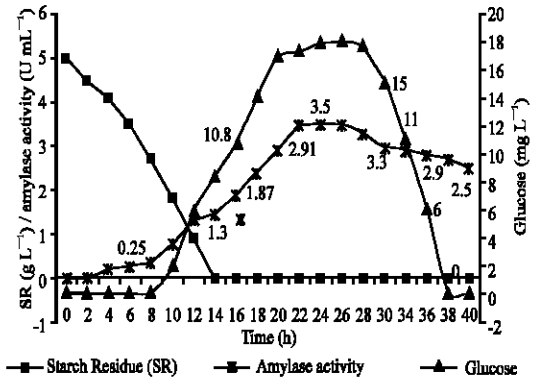

Fig. 3: Time course of starch degradation, extracellular amylase secretion and glucose production from soluble starch by *C. aerius* growing in starch medium (5 g L^{-1}) at 30°C

Table 2: Starch degradation by *C. aerius* in the presence of glucose

Reside	Glucose (g L^{-1})	Starch (g L^{-1})	Starch residue (6 h)	Starch (24 h)
Experiment No. 1	0	10	8.4	0
Experiment No. 2	0.2	10	9.4	0
Experiment No. 3	0.5	10	9.8	0
Experiment No. 4	1	10	10	1.3

shown in Table 2, as the results indicated, there was no amylase activity in growth media contained 1 g L^{-1} glucose as sole carbon source and starch degradation was inhibited in media contained 10 g L^{-1} starch plus different amount of glucose as carbon source, while starch could induce amylase production by *C. aerius*. Previous Studies have shown that starch degradation and amylase production by different microorganisms, such as *Aspergilluus* sp.[23] or budding yeast such as

Table 3: Characteristic of SCP produced by monoculture and coculture (initial starch concentration was 30 g L^{-1})

C. aerius inocolum (%v/v)	*S.cervisiae* inoculum (% v/v)	Starch residue (g L^{-1}) after 36 h	Total cultivation time (h)[a]	Final biomass (g L^{-1})`	Protein content (%)	Efficiency of biomass production (%)
2	0	12.2	>96	9.89	28.3	83.5
	2	7.3	>96	11.15	33.7	94.1
	5	5.2	>96	11.21	43.1	94.6
	10	24.5	>96	11.23	42.6	94.8
5	0	4.3	78	10.02	27.1	84.7
	2	0	70	11.13	42.3	94.3
	5	0	58	11.22	44.2	94.7
	10	0	56	11.23	45.1	94.8
10	0	0	64	9.93	27.8	83.7
	2	0	56	11.18	45.8	94.4
	5	0	56	11.22	44.2	94.7
	10	0	56	11.26	44.1	95.1

[a]Total cultivation time, was estimated when total concentration of reducing sugar (liberated from starch) was 0 (mg mL^{-1})

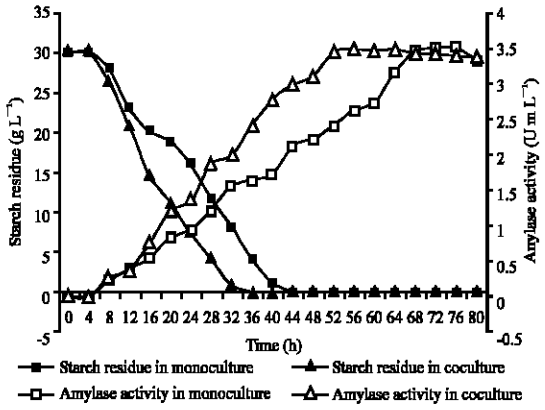

Fig. 4: Time course of starch degradation and extracellular amylase secretion in monoculture and coculture at 30°C

Endomycopsis fibuligera[20] *Saccharomyces cerevisiae* var. *diastaticus*[24] and *Aureobasidium pullulans*[25] is subject to severe feed back inhibition by glucose. The two main factors controlling amylase synthesis in microorganisms are induction and catabolite repression. Catabolite-inducible enzymes are produced in the presence of either their substrate in surrounding medium or compound structurally related to them. Starch, the substrate of amylases has a high molecular mass and cannot pass though cell membrane directly, hence cells receive signals for accelerated synthesis by means of low molecular mass fragments, using high concentration of starch in media, causes high concentration of these low molecular mass and rapid metabolism of them in cells producing D-glucose and subsequent catabolite repression. A number of experimental strategies may be investigated to overcome this type of problems, these include supplying the inducer continuously at low rate, using slowly metabolized derivates or supplying relatively inert synthetic thio analogues [26], as the aim of this study was to use only starch as substrate, we tested the coculture hypothesis (Dual fermentation)[27]. According to the pervious studies, cocultures of two microorganisms in starch containing media would prevent accumulation of inhibitory concentration of reducing sugar and this would result in an enhancement of the amylolytic activity, the amount of starch metabolized and finally an increase in starch degradation rate[2,23,27]. As the results indicated (Fig. 4) in a soluble starch media (30 g L^{-1}) containing 5% (v/v) *S. cerevisiae* plus 5% (v/v) *C. aerius* comparing to mono culture of *C. aerius* (5%v/v) there was a regular increase in amylolytic activity and the time needed for 30 g L^{-1} starch degradation is much more shorter than monoculture of *C. aerius*.

SCP production by coculture: We then tested the effect of increasing the concentration of yeast inoculums on the length of cultivation and final biomass production. The results indicated that as the concentration of *S. cerevisiae* inoculum increased in each level of *C. aerius* concentration, the time required for the completion of cultivation decreased dramatically (Table 3), infact when *C. arerius* concentration was 2% (v/v) due to low level of amylase secretion, cultivation time is too long (>96 h); for example, using a 5% (v/v) *C. aerius* inoculum and 5%(v/v) *S. cerevisiae* inoculum, raw starch was completely utilized in 34-36 h. As the data indicated (Table 3), in appropriate concentration of *C. aerius* (>2% v/v), the starch degradation rate and the release of reducing sugar by amylolytic enzyme system of *C. aerius* wasn't the limiting step, instead the rate of assimilation of sugars by yeasts appeared to be the limiting factor, therefore by increasing in *S. cerevisiae* inoculum which can only assimilate reducing sugars we could overcome this problem. The above results are in agreement with pervious studies that shows increasing the level of yeast inoculum greatly decrease the fermentation time[2,28]. It is noteworthy that when 2-10% (v/v) *S. cerevisiae* inoculum were used the final protein content and biomass were comparable; it was only the length of cultivation that was markedly effected.

Since the rapidness of starch degradation and yield of high biomass with high protein content are economically desirable in industry, it is suggested that 5% v/v of both yeast are used in further investigations.

The results of this investigation clearly show that simultaneous fermentation of raw starch to SCP by a mixture of starch-digesting yeast (*C. aerius*) and a non-starch-digesting yeast such as *S. cerevisiae* is feasible. The efficiency of starch digestion and assimilation by coculture was >94% comparing to media contained glucose. Use of such a synergistic combination of organisms allows elimination the enzymatic starch hydrolysis step as currently used in many commercial processes from starchy biomass.

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REFERENCES

1. Lehninger, A.L., 2000. Principle of Biochemistry. Worth Publishers, Inc. New York, pp: 287-288.
2. Abouzied, M.M. and C.A. Reddy, 1986. Direct fermentation of potato Starch to ethanol by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae*. Applied Environ. Microbiol., pp: 1055-1059.
3. Nigam, P. and D. Singh, 1995.Enzyme and microbial systems involved in starch processing. Enz. Microbiol. Technol., 17: 770-778.
4. Tuse, D., 1984. Single cell protein: Current status and future prospects. Crit. Rev. Food Sci. Nutr., 19: 273-325.
5. Waites, M.J., N.L. Morgan, J.S. Rockey and G. Higton, 2001. Industrial Microbiology: An Introduction. BlackWell Science Ltd. London, pp: 219-226.
6. Pandy, A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Sigh and R. Mohan, 2000. Advances in microbial amylases: Review article. Biotechnol. Applied Biochem., 31: 135-152.
7. Ray, R.R. and G. Nanda, 1996. Microbial β-amylases: Biosynthesis characteristics and industrial application. Crit. Rev. Microbiol., 22: 181-199.
8. Walker, G.M., 2000. Yeast Physiology and Biotechnology. John Wiley and Sons. England., pp: 54.
9. Laluce, C. and J.R. Matton, 1984. Development of rapidly fermenting strains of *Saccharomyces diastaticus* for direct conversion of starch and dextrins to ethanol. Applied Environ. Microbiol., 48: 17-25.
10. Kondo, A., H. Shigechi, M. Uyama, T. Matsumoto, S. Takahashi, M. Ueda, A. Tanaka, M. Kishimoto and H. Fukuda, 2002. High level ethanol production from starch by a flocculent *Saccharomyces cerevisiae* strain displaying cell-surface glucoamylase. Applied Microbiol. Biotechnol., 58: 291-296.
11. Linardi, V.R. and K.M.G. Machado, 1990. Production of amylase by yeasts. Can. J. Microbiol., 36: 751-753.
12. Macfaddin, J.F., 2000. Biochemical Tests for Identification of Medical Bacteria. 3rd Edn. Lippincott Williams and Wilkins, pp: 412-423.
13. Bernfeld, P., S.P. Colowick and N.O. Kapan, 1995. Method in Enzymology. Academic Peress, New York, pp: 36-44.
14. Wilson, J.J. and W.M. Ingledew, 1982. Isolation and characterization of *Schwaniomyces alluvius* amylolytic enzymes. Applied Environ. Microbiol., pp: 301-307.
15. Jorgensen, H., L. Olsson, B. Ronnow and E.A. Palmqvist, 2002. Fed-batch cultivation of baker's yeast followed by nitrogen or carbon starvation: Effect on fermentative capacity and content of trehalose and glycogen. Applied Microbiol. Biotechnol., 59: 310-317.
16. Scopes, R.K., 1994. Protein Purification: Principle and practice. Springer Verlag New York, INC., pp: 349-350.
17. Kurtzman, C.P. and J.W. Fell, 1998. The Yeast (A Taxonomic Study). Elsevier.
18. Iefuji, H., M. Chino, M. Kato and Y. Iimura, 1996. Raw starch digesting and thermostable α-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. Biochem. J., 318: 989-996.
19. Wanderley, K.J., F.A. Torres, L.M. Moraes and C.J. Ulhoa, 2004. Biochemical characterization of α-amylase from the yeast *Cryptococcus flavus*. FEMS Microbiol. Lett., 231: 165-169.
20. Clementi, F., J. Rossi, L. Costamagna and J. Rosi, 1980. Production of amylase(s) by *Schwanniomyces castellii* and *Endomycopsis fibuligera*. Antonie Van Leeuwenhoek. 46: 399-405.
21. De Mot, R. and H. Verachtert, 1985. Purification and characterization of extracellular amylolytic enzyme from yeast *Filobasidium capsuligenum*. Applied Environ. Microbiol., 50: 1474-1482.
22. Berry, D.R., I. Russell and G.G. Stewart, 1987. Yeast Biotechnology. Allen and Unwin. London, pp: 289-293.

23. Reddy, C.A. and M.M. Abouzied, 1986. Glucose feedback inhibition of amylase activity in *Aspergillus* sp. and release of this inhibition when cocultured with *Saccharomyces cerevisiae*. Enz. Microbiol. Technol., 8: 659-664.
24. Pretorius, I.S., M.G. Lambrechts and J. Marmur, 1991. The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: An overview. Crit. Rev. Biochem. Mol. Biol., 26: 53-76.
25. Lodato, P.B., F. Forchiassin and M.B. Segovia de Huergo, 1997. Amylase production by *Aureobasidium pullulans* in liquid and solid media. Rev. Argent. Microbiol., 29: 115-21.
26. Goto, C.E., E.P. Barbosa, L.C.L. Kistner, F.G. Moreira, V. Lenartovicez and R. Peralta, 1998. Production of amylase By *Aspergillus fumigates* utilizing α-methyl-D-glucoside, a synthetic analogue of maltose, as substrate. FEMS Microbiol.Lett., 167: 139-143.
27. Casida, J.R., 1991. Industrial Microbiology. Willey Eastern Limeted. India, pp: 161-175.
28. Nagodawithana, T.W. and K.H. Steinkraus, 1976. Influence of the rate of ethanol production and accumulation on the viability of *S. cerevisiae* in rapid fermentation. Applied Environ. Microbiol., 31: 158-162.