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High performance amylolytic yeast strains isolation and identification for valorization of potatoes waste available in Burkina Faso

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Abstract

This study deals with isolation and identification of high amylolytic activity strains from potato wastes for biomass production. A total of twenty five (25) yeast strains were isolated using standard microbiological methods. Ten (10) strains were retained on the basis of their capacity to hydrolyze starch. Identification to species level of selected strains was performed with an API 20C AUX (Bio-Mérieux) according to the manufacturer's instructions. Strain NOY 1 was identified as *Candida utilis* and had the best diameter of starch hydrolysis (24 mm) and the optimal enzymatic activity (1248 $\mu M.l^{-1}.mn^{-1}$). Optimal growth parameters of this strain were 30°C, pH 5; under these conditions, 5 g of starch/liter of medium were used for biomass production in an incubator at 150 rpm for 72 hours. The maximal biomass and protein content were obtained after 36 and 40 hours of incubation and were 5.65 g/l of medium and 0.53 g/g of dry weight respectively.

Introduction

Yeasts are an attractive group of lower eukaryotic microorganisms. Some of them are used in several industrial processes that include brewing, baking and production of a variety of biochemical compounds. Recently, yeasts have been developed as host organisms for the production of foreign proteins. *Saccharomyces cerevisiae* has usually been the yeast of choice, but an increasing number of alternative non-*Saccharomyces* yeasts have now become accessible for modern molecular genetics techniques (Dominguez *et al*., 1998). In recent years increasing attention has been given to the conversion of food processing wastes into valuable by-products such as the production of yeast protein from potato (Skogman, 1976) and confectionery effluents (Forage, 1978). The recovery of such by-products can significantly reduce the rate of waste disposal and contribute to the cleaning up of the environment. Several microbial processes for treatment of potato wastes have been described, mainly with the objective to reducing the Biochemical Oxygen Demand (B.O.D.) of the effluents. Little attention has been given to the production of a useful by-product. Rieserc (1954) recovered up to 50% of the dissolved solids as yeast when he grew *Candida utilis* on effluents from a potato starch factory. Bloch *et al*. (1973) grew the amylolytic *Aspergillus foetidus* on alkaline peel waste neutralized and supplemented with ammonium and phosphate salts. He then used the medium after the growth of this organism to saccharify further waste fermented by *Saccharomyces cerevisae* to produce ethanol. The main component of the potato tuber is starch (80 per cent of the dry solids and 20 per cent of the total mass of the potato). Amani *et al*. (2003) have reported that potatoes contain about 72% of starch. The transformation of these tuber wastes with high level of starch into microbial biomass requires amylolytic strains with strong potential to hydrolyze starch.

The main objective of this study was to isolate, identify and select high performance amylolytic yeast strains from potato residues available in Burkina Faso for later valorization of these residues. Additionally optimal enzymatic hydrolysis parameters (i.e., enzyme to substrate level, temperature and pH), starch concentration and protein content of yeast strain were determined.

Material and Methods

Sampling

Thirty (30) samples of potato wastes were collected from waste dumping sites in the three potato production areas (High basins, Western

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Center, Northern Center) of Burkina Faso during the month of January to February 2011 in sterile stomacher sachets and brought to the laboratory. The three areas constitute the largest producing areas of sweet potato in Burkina Faso. However we noted a selling problem of these tubers and the absence of processing industries for these tubers worsens the problem of storage. Thus each year producers record significant food losses. Part of the waste is consumed by animals while a lot more gets rotten and contributes to environmental pollution. Thus large quantities of waste are available in these areas and were the object of our sampling. On the whole 30 samples from domestic activities (10 from each area) were collected for this study.

Yeast strains isolation

Yeast strain isolation was performed with SABOURAUD CAF AGAR medium (Liofilchem, Italy; REF 610203). Ten (10) g of each sample was put directly into a 250 ml flask containing 90 ml of sterile SABOURAUD CAF liquid medium. After 72 hours of incubation at 30°C in a shaker at 150 rpm, the presence of yeasts cells in media was examined using light microscopy and 0.1 ml of the culture was shown on the surface of the solid Sabouraud medium after several adequate dilutions with physiological water (0.85 g/L NaCl).

Selection and purification of amylolytic strains

The culture medium used for the selection of amylolytic yeast strains was a semi synthetic medium containing only starch as source of carbon and energy (Lagzouli *et al*., 2007a, 1972; Mellouli *et al*., 2003-2004). The media (broth and agar medium) were sterilized by autoclaving for 15 minutes at 121°C, initial pH being fixed before sterilization at 5 with HCl 0.1 N. Chloramphenicol (0.5g/l) and sugars sterilized by filtration with Millipore filter (0.45 µm) were aseptically added. Yeasts strains isolated on SABOURAUD CAF AGAR were mended in the semi synthetic broth. After 24 hours incubation at 30°C the yeast isolates were streaked on the surface of the solid Sabouraud medium. It was incubated for 48 hours at 30°C. Starch degradation was detected by the disappearance of the blue colour of the medium around the microbial colonies after addition of iodine solution. Colonies with the largest halo-forming zone were isolated and selected for further investigation. Evaluation of the clear zones was estimated as diameter (mm) of the clear zone. Yeast colonies producing large clear zones were picked up and purified three times by streaking on Potato Dextrose Agar (PDA). Pure cultures were maintained on PDA, stored in a refrigerator at 4°C and sub-cultured at 3 month intervals.

Identification of yeasts strains

Yeast strains were identified based on the morphological, physiological and cultural characters as described by Barnett *et al*. (1983) using the yeast identification software of Barnett *et al*. (1996). The identification was based on different biochemical tests including the fermentation of sugars, assimilation of carbon compounds, growth at 37°C and 40°C. Identification of amylolytic strains was completed using API 20C AUX (Biomerieux, REF 20 210) according to the manufacturer's instructions. The corresponding API LAB Plus database and software were used for presumptive identification of yeast species.

Optimization of the growth of Candida utilis *NOY1*

Effect of temperature and medium pH on yeast growth

To ascertain the effect of culture conditions the present study was carried out at different temperatures (20; 25; 30; 35 and 40°C), and medium pH (3; 3.5; 4; 4.5; 5; 5.5; 6; 6.5; 7; 7.5 and 8.). Their effects on biomass production were recorded. Cultures were incubated at 30°C at 150 rpm for 48 hours for pH effect determination and pH 5 at the same condition for temperature effect (Lagzouli *et al*., 2007a).

Effect of the starch concentration

The effect of the starch concentration was tested on the semi-synthetic medium with 2.5; 3.5; 5; 7.5; 9 and 10 g/L of starch and initial medium pH 5 at 30°C. Effect of carbon source on biomass yield was recorded. Fermentation experiments were carried out for 72 h at 150 rpm.

Determination of α-amylase activity

The fermented broth was centrifuged at 7000 rpm for 10 min after the 75 hours incubation, and substrate-free supernatant was used for estimation of enzyme activity. Amylase activity was determined by measuring the reducing sugar formed by the enzymatic hydrolysis of starch. The protocol of Somogyi (1952) and Nelson (1944) was used. In this protocol, 0.25 ml soluble starch (1%), 0.15 ml phosphate buffer (0.1 M) and 0.1 ml enzyme solution were mixed and incubated at 40°C in a water bath for 30 min. The reaction was stopped with 2 ml of Somogyi reagent, and 1.5 ml of distilled water, followed by boiling for 15 min to develop blue color. The absorbance was measured at 540 nm with a spectrophotometer µQuant (BIO-TEK INSTRUMENTS, INC). The blue color was measured against the control in which no enzyme was added.

A calibration curve of absorbance and concentration of glucose was established with known amounts of glucose. One unit (µmol/L/min) of glucoamylase was defined as the amount of 1 µmol of reducing sugar per liter of enzymes per min, measured as glucose under the conditions of assay.

Batch fermentation in a laboratory flask in optimal conditions

Candida utilis NOY1 was grown in a 2 litre flask containing 900 mL of the production medium (5 g/l starch; 3 g/L KH_2PO_4; 0.77 g/L CH_4NO_2; 3 g/L yeast extract) and 1 ml of oligoelements solution (Cooney and Levine, 1972; Mellouli *et al*., 2003-2004). The initial pH of the medium was adjusted to 5 with 0.1 M of HCl before sterilization. The inoculum was prepared in Erlenmeyer flasks in a volume corresponding to 10% of fermentation broth medium and incubated at 30°C at 150 rpm (Lagzouli *et al*., 2007b). The flask was incubated at 30°C in a flask on rotary shaker set at 105 rpm for 72 h. Ten(10) ml of the sample was analysed at 4 hour intervals for residual glucose, starch concentration, cell biomass and protein of dried biomass.

Dry cells, Nitrogen content and residual starch analysis

The concentration of yeast cells in the fermenting mash was measured by the turbidimetric (absorbance at 600 nm) method and by determining dry weight of yeast cells (Lagzouli *et al*., 2007b). Dry cell mass was determinated gravimetrically. Cells were harvested by centrifugation at 10000 rpm for 10 min, washed twice with distilled water and dried in an oven at 105°C for 24 hours. The dried cells were weighed and kept for protein content determination. Nitrogen content of yeast biomass was determinated by microkjeldhal method (AOAC, 1990). The crude protein values were obtained by multiplying the nitrogen content by 6.25 (Mateles and Tannenbaua, 1968).

The culture supernatants were collected for determination of sugar consumption in the medium. Residual sugars were determined as glucose by the colorimetric method using 3.6 dinitrosalicylic acids (DNS): 1 ml of diluted supernatant left after the separation of the biomass was mixed with 1 ml of dinitrosalicylic acid and heated to 100°C for 5 min. The reaction was stopped in ice and optical density was read at 540 nm with spectrophotometer µQuant (BIO-TEK INSTRUMENTS, INC) against a glucose standard graph (Miller, 1958). For starch concentration determination, the supernatant was mixed with I_2/KI and incubated for 10 minutes before the measurement of the optical density at 580 nm. The content of starch was given by using a standard curve obtained with a 5 mg/ml starch solution (Jarvis and Walker, 1993; DeMan, 1980).

Statistical analysis

Fermentation and all laboratory analysis were done in triplicate. The data was subjected to Duncan's Multiple Test using the Statistical Package for Social Science (SPSS Inc., Illinois USA, Version 17), and $p<0.05$ was considered statistically significant (Duncan, 1955).

Results

Isolation, identification and selection of amylolytic yeast strains

Twenty five (25) yeasts strains were isolated. Among them, ten (10) hydrolyze starch and were considered as amylase producing strains. These strains were identified using API gallery 20 C AUX kit (Biomérieux REF 20 210). Strain NOY1 was identified as *Candida utilis* and was selected for the present work. The main characteristics of the eight amylolytic yeasts are presented in Table 1. The enzymatic activity and the diameter of the starch hydrolysis of different strains are also presented.

Amylolytic activity is a function of yeast strains and varies from 12 to 1248 $\mu M.l^{-1}.min^{-1}$. *Candida utilis* NOY1 had the best enzymatic activity (1248 $\mu M.l^{-1}$. Min^{-1}) and the largest diameter of hydrolysis (24 mm).

Optimization of the growth of Candida utilis *NOY 1* ***pH and temperature effect on*** **Candida utilis** ***NOY 1 growth***

The pH of growth medium plays an important role in the production of biomass and microbial enzymes. In the present study, maximum production was achieved using a medium with an initial pH of 5 (Figure 1a). Temperature also influences biomass production, and yeast biomass increased progressively with increase in temperature from 25°C reaching a maximum at 30°C (Figure 1b). Above 35°C, there

Table1. Identification, Enzymatic activity and Hydrolyze diameter of the ten amylolytic yeast strains selected

Yeasts isolates	Enzymatic Activity ($\mu M.l^{-1}.min^{-1}$)	Hydrolyze Diameter (mm)	Identification
NOY 1	1248	24	*Candida utilis*
NOY 2	575	13	*Candida guilliermondii*
NOY 3	774	15	*Trichosporon mucoides*
NOY 4	125	5	*Candida guilliermondii*
NOY 6	36	3	*Candida pelliculosa*
NOY 7	345	11	*Candida lusitaniae*
NOY 8	1192	19	*Candida guilliermondii*
NOY 9	12	<1	*Candida ciferrii*
NOY 10	547	13	*Candida famata*
NOY 12	658	15	*Trichosporon mucoides*

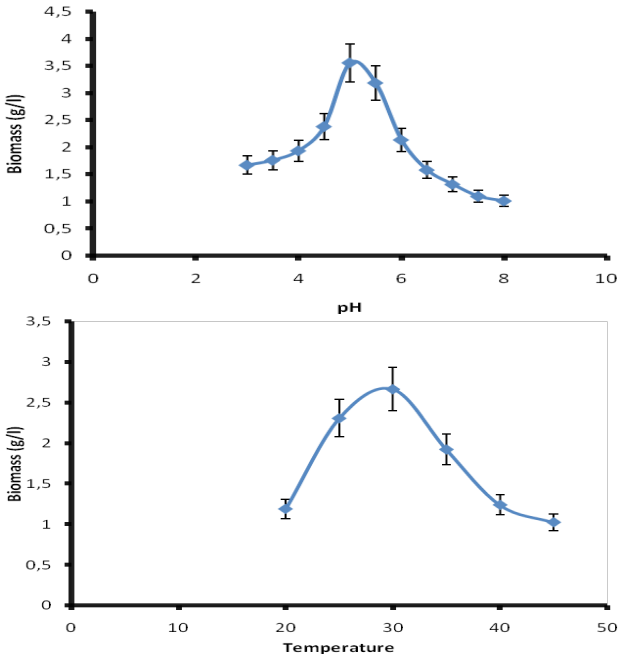

Figure 1. Effect of pH (a) and temperature (b) on the production of yeast biomass of *Candida utilis* NOY 1.
Error bars show standard deviation among three observations. Experiments were carried out at 30°C and 150 rpm and incubated in different pH. Values are means of triplicates ± S.D. (Standard Deviation).

Table 2. Effect of starch concentration on the specific growth rate, initial and final biomass and yield of strain identified as Candida utilis NOY 1 in batch culture

So (g/l)	µm (h^{-1})	Xi (g/l)	Xf (g/l)	$Y_{x/s}$(g/g)
10	0.19	1.21	5.65	44.3
9	0.28	1.14	5.21	45.22
7.5	0.35	1.18	4.98	54.28
5	0.48	1.25	4.5	64.24
3.5	0.47	1.24	3.32	59.42
2.5	0.41	1.09	2.12	51.5

S0: initial starch concentration that varied from 2.5 to 10 g/l, µm: maximum speed of growth, Xi: initial biomass, Xf: Final biomass obtained after centrifugation and drying of culture, Yx/s: Yield of product from the biomass (g of biomass per g of consumed sugars). All the experiments were carried out at 30°C, pH 5 and 150 rpm with addition of different starch concentration and during 24 hours.

was a reduction in the yeast biomass.

Effect of starch concentration on Candida utilis NOY 1 growth

Yeast biomass increased in relation to the increase in starch concentration from 2.5 g/L to 5 g/L. Above 5 g/L of starch concentration; there was a decline in biomass production (Table 2). Also, growth of the studied yeast increased according to the concentration of starch to reach a maximum value at 10 g/L. The initial and final biomass was used for the determination of the total biomass produced during fermentation. The maximum amylolytic activity was obtained with the best yield of biomass production and was 1248 µM.l^{-1}.mn^{-1} at 5 g/L of starch. Yield of biomass production and yeast maximum growth speed were optimal (64.24 % and 0.48 h^{-1}., respectively) at the concentration of 5 g/L of starch.

Biomass production and analytical procedures of Candida utilis *NOY 1 in medium containing starch as only source of carbon*

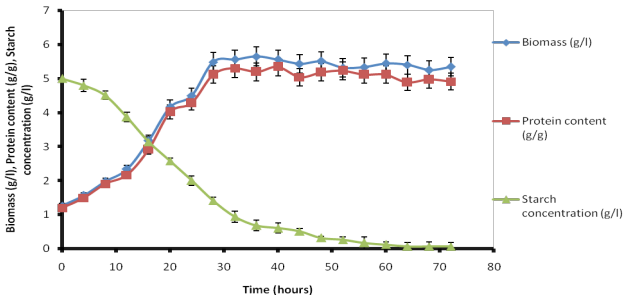

Figure 2. Time course of microbial biomass protein production and starch hydrolysis by *Candida utilis* NOY 1. All the experiments were carried out at 30°C, pH 5 and 150 rpm with 5g/L of starch. Fermentation was led 72 hours. Error bars show standard deviation among three observations. Values are means of triplicates ± S.D. (Standard Deviation). The protein content is given in g of protein per 10 g of dried biomass.

Biomass protein and starch concentration are presented in Figure 2. Biomass is given in g/L of culture medium and increased with time during the first 30 hours to reach a maximum of 5.65 g/L. The protein content of cell biomass was 0.534 g of protein per g of dried yeast; what corresponds approximately to a proteomics rate of 53 %.

Discussion

The API gallery 20C allowed identification of 10 amylolytic yeast strains isolated from potato residues. However, this identification proved to be insufficient especially since the produced proteins will be destined for human nutrition. It will thus be necessary to avoid using pathogenic germs. This fact molecular identification would have provided more precision enzymatic activities were similar to those found in literature by other authors with other enzymes. Bousmaha *et al*. (2007) found a β-fructofuranosidase activity of 1538 µM.l^{-1}.mn^{-1} for the *Candida guilliermondii* L2 strain with 5g/L of *saccharose*, after 24 hours at 30°C in a liquid semi-synthetic medium. However our values appear lower compared to those found by Lagzouli *et al*. (2007b). These authors found a maximum alpha-amylase activity of 1742 µM.l^{-1}.mn^{-1} after 72 hours of culture with 5 g/l of starch using a strain of *Candida guilliermondii* LGZ 14. In effect strains of *Saccharomyces cerevisiae* J1, *Candida krusei* J2 and *C. krusei* J3 were isolated in fermented bamboo shoot product from villagers in Amphur Kokpho by Maneesri and Masniyom (2007). Also production of amylase by *Aspergillus* sp. was reported by Rahman *et al*. (1993) and Cherry *et al*. (2004). Lagzouli *et al*. (2007b) isolated a yeast strain (*Candida famata*) from traditional Moroccan sourdough able to produce amylase. In Burkina Faso, yeasts (*Saccharomyces* and *Schizosaccharomyces*) are already isolated from

mango waste and used for alcohol production (Somda *et al*., 2010). Our results indicate for the first time that yeasts such as *Candida* capable of hydrolyzing starch and can be used for single cell protein production.

The optimal pH of biomass production is in agreement with published data. Yeasts grow in a broad range of pH from 2.4 to 5.8, with an optimal value between 4 and 5 (Oteng-Gyang, 1984). Similarly, Quang *et al*. (2000) found optimum pH of 4.9 for fermentation medium of *Thermomyces lanuginosus* ATCC 34626. Similarly, Reiser and Gasperik (1995) reported that optimum biomass production and glucoamylase activity of *Saccharomycopsis fibuligera* was observed at pH 5.5. The pH affects yeast membrane permeability and thus the growth of cells.

Similar results were reported by Rene and Hubert (1985) with *Filobasidium capsuligenum* glucoamylase activity production. *Candida guilliermondii* presents an optimum of growth and production of fructofuranosidase at 30°C (Bousmaha *et al*., 2007). Statistical analysis of data from Figure 1a and 1b pointed that yeast biomass was significantly different ($p \leq 0.05$) under different culture media pH and temperature conditions.

Results reported on starch effect in biomass production showed that beyond 5 g/L, starch inhibits yeast growth and amylolytic activity in agreement with other authors (Aiba *et al*., 1965; Lehninger *et al*., 1994). The enzymatic receivers could be saturated. At one lower concentration, yeasts develop slowly because less substrate is placed at their disposal (Bousmaha *et al*., 2007).

The amylolytic activity is a function of the microbial growth. The higher the growth of yeast, the greater the amylolytic activity. Similar starch concentration (5 g/L) was used for the production of biomass and amylase by *F. capsuligenum* (Rene and Hubert, 1985), *Clostridium thermosulfurogenes* (Hyun and Zeikus, 1985) and *Clostridium* sp. (Madi *et al*., 1987). On the contrary, other investigators reported that maximum amylase and biomass production was produced at 10 g/L, with *Lipomyces kononenkoae*, (Isabel, 1982), *Schwanniomyces alluvius* (Jeffrey and Michael, 1982) and 15 g/L with *Chaetomium thermophilum* (Jing *et al*., 2005). The optimization of *Saccharomyces cerevisiae* growth parameters made it possible to improve the growth and production of biomass proteins. Also this strain could be a source of amylolytic enzyme which will find its application in industry.

Results reported in Figure 2 show that protein content was related to the cell multiplication, it decreases until the cells become exhausted in the medium. The initial pH of the nutrient solution was adjusted to 5. Statistical analysis of data from Figure 2 pointed that yeast biomass were significantly different ($p \leq 0.05$) under different culture conditions.

The results showed that pH, temperature and starch concentration have an important effect on biomass protein and amylase production. Cell proteins increase proportionally with biomass production. In parallel starch is gradually consumed in the medium during growth and its hydrolysis is noticeable on a logarithmic phase. Starch concentration starts to become limiting after 40 hours of fermentation. The level of crude protein (53%) obtained from the dry biomass suggests that the product is a potential food and feed supplement when compared to the lower limits of 8% for cattle and poultry feed (Han et Anderson, 1974).

Conclusion

The present results add to current knowledge of microbial biomass protein production by yeasts culture fermentation from waste products. The research indicated that amylolytic yeasts such as *Candida utilis* and *Candida guilliermondii* are very important for optimizing yeast biomass production using agricultural waste products that are rich in starch. These strains can also be used in industry to produce amylase and other very important enzymes. Thus these amylolytic yeast strains could be used for a later valorization of the residues of tubers which are extremely rich in starch and which are available in Burkina Faso.

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