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ISOLATION, CHARACTERIZATION, FERMENTATION STUDIES OF THERMOTOLERANT AMYLOLYTIC YEAST

Amina Fatima[2], Syed Shah Minallah Alvi[2], Gudidevuni Sheshank Bushan[1] and Dr. Chand Pasha[1]*

1. Department of Microbiology, Nizam college, Osmania University, Hyderabad, Telangana, India.

2. Department of Microbiology, Mumtaz Degree and PG college, Malakpet, Hyderabad.

Email: cpasha21@gmail.com

ABSTRACT

Amylolytic and Thermotolerant Yeast was isolated from bakery samples and soil samples from cooking area. The isolate was confirmed as *Candia tropicalis* from colony morphology, microscopic observations and biochemical tests. Yeast is growing and the amylase of isolate is also active at 42°C. The isolate *C. tropicalis* was resistant to azoles and Echinocandin drugs and susceptible to Amphotericin B. Soluble starch and Rice starch was used as substrate for ethanol fermentation. The rice starch was obtained from boiled rice and alkaline treatment. The substrates were hydrolysed by commercial amylase and Amylolytic *C. tropicalis* isolate. Commercial enzyme yielded 71 ±0.98 % and 70 ± 1.22 % hydrolysis for Soluble starch and Rice starch. Hydrolysis of Soluble starch and Rice starch by Simultaneous Saccharification and Fermentation studies yielded 87.1±0.2% and 85.2±0.9% of substrate hydrolysis. The isolate is reported to have 87.29±0.9% and 86±0.9%. of ethanol fermentation efficiency for soluble starch and rice starch at 10 grams level. Separate saccharification and fermentation yielded ethanol fermentation efficiency of 89.3±1.1% and 88.7±1.3% for soluble starch and rice starch with hydrolysis of 87.1±0.2% and 85.2±0.9%. The study claims to perform Simultaneous Saccharification and Fermentation studies using isolated amylolytic and thermotolerant *C.tropicalis* for efficient production of Bioethanol. This can be an efficient bioprocessing strain as ethanol production is carried out at higher temperatures proving mesophilic enzyme ineffective.

Keywords: Amylolytic Yeast, Thermotolerant Yeast, Bioethanol, Simultaneous Saccharification and Fermentation.

1. Introduction

In present times, enzymes use has been escalated rapidly leading to evolution of biotechnology and industrial microbiology due to their catalytic activity for biochemical reactions (Maghraby et al., 2023). The demand for enzymes with greater activity, specificity and stability is increasing for optimal industrial applications (Victorino da Silva Amatto et al., 2022). Amylase are among the most extensively studied enzymes and are prominent in brewing, detergent, tannery, paper, biofuel, food industry etc (Farooq et al., 2021).

Demand for industrial enzymes is growing steadily and expected to reach more than 8 billion dollars in 2026 (Research B et al., 2021). Most amylases cannot tolerate harsh industrial conditions as industrial processes work under extreme pH, temperature and high substrate concentration (Paul et al., 2021). Most enzymes are mesophilic in origin and have stability issues, enzymes extracted from thermophilic organism are exceptional to confer stability and to retain biological activity (Arbab et al., 2022). Starch saccharification is more efficient with thermostable amylase than conventional amylase of mesophilic origin (Kikani et al., 2022).

The world is facing ever growing population and global economy leading to increased usage of energy and exhausting the available resources by increased waste production, many countries are now planning to meet the energy and waste management needs by employing biofuel as a safer economical decision for sustainable development (Hsu et al., 2024). Bioethanol is one of the most produced biofuels and produced by microbial fermentation of carbohydrates from plants or algae (Ambaye et al., 2021). Annually 17% of food is wasted at consumer levels and most food contains starch in it; starch can be degraded by amylase and the glucose released can be used for various bioprocesses including bioethanol production (Lu et al., 2023). In recent years, bioethanol is produced from plant biomass from thermophilic microbes and their enzymes and stated to have advantages over mesophilic bacteria and counterparts (Mesbah et al., 2022).

The bioethanol production can be also done by simultaneous saccharification and fermentation in which starchy substrate is hydrolysed and fermented simultaneously by one organism, this method if focused and optimized can greatly reduce production cost of bioethanol (Derman et al., 2022). Simultaneous Saccharification and Fermentation is highly dependent on raw materials and many extrinsic factors like organism, enzymes and pH (Devos et al., 2022). A well noted drawback of Simultaneous Saccharification and Fermentation is incompatibility of temperature and pH optima for the hydrolysis and fermentation steps, Thermophilic organisms and thermophilic enzymes can pave a major milestone towards Simultaneous Saccharification and Fermentation processes as it works best at 40-50°C and at pH 4.5-5.5 (Chacon et al., 2021).

The present study focuses on isolating a microorganism with thermotolerant and amylolytic activity for Simultaneous saccharification and fermentation of starch to efficiently produce bioethanol at higher temperatures.

2. Materials and Methods

a – Amylase was purchased from HiMedia and the isolated *C. tropicalis* strain was maintained in our laboratory at 42°C.

2.1 Sample collection and Preparation

Bakery samples and soil samples near cooking area was collected to obtain yeast with amylolytic and thermotolerant nature. The samples were collected in aseptic bag and suspended in normal saline and diluted. The samples were enriched in modified YEPS containing 1% Yeast extract, 2% Peptone and 2% Starch and incubated ranging at temperatures ranging from 38°C to 45°C at 100rpm shaking for 3 days.

2.2 Screening for Amylolytic and thermotolerant yeast

The enriched samples were collected from 38-45°C to screen for thermotolerant species and spread on YEPS to screen amylolytic yeasts. The samples were incubated at 42°C for 2 days and screening for amylase activity is done.

The amylase activity is confirmed by pouring ringers iodine solution on grown plates containing yeast to select the yeast with amylolytic activity by the zone of hydrolysis. The yeast isolates with amylolytic activity and thermotolerant activity was screened and sub-cultured for further studies.

2.3 Colony characterization and colony morphology of Amylolytic and thermotolerant yeast

The isolated yeast was grown on YEPS media and colony morphology and microscopy colony characteristics were observed to confirm the isolate species. The grown petri plate was observed for colony morphological studies. Simple staining was performed for isolates and observed under 100X oil immersion and colony characteristics were recorded.

2.4 Biochemical characterization of Amylolytic and thermotolerant yeast

Further confirmation of the isolate was done by performing various biochemical tests, like urease test and Sugar assimilation test. For sugar assimilation tests of glucose, sucrose, starch, xylose, lactose, maltose, galactose, trehalose, arabinose, cellobiose, Sorbitol, Inositol, Erythritol and Glycerol were performed. The isolate was grown in minimal media with specific sugar and tested for fermentation of different sugars by subculturing the isolate on basal sugar medias of different sugar and incubated for 24 hours at 42°C and pH was recorded.

2.5 Antimicrobial drug resistance

The antibiotic susceptibility was carried out by spreading 100µl actively growing isolate on YEPS plates, diffusion assay was performed on isolate against 10mg discs of Fluconazole, Voriconazole, Itraconazole, Posaconazole, Caspofungin, Micafungin and Amphotericin B. The plates were incubated at 42°C for 24hrs to observe the antibiotic susceptibility profile of isolate.

2.6 Isolation and Subculture of Amylolytic and Thermotolerant Yeast species

The confirmed isolate was sub-cultured and streaked into YEPS plates and incubated for 48hrs at 42°C and obtained pure colony plates were stored at 4°C and revived for further fermentation studies.

2.7 Rice starch Extraction

The ethanol production and fermentation efficiency were studied by 2 substrates. Rice starch and Soluble starch were selected for fermentation studies for bio ethanol production.

The rice starch was obtained from the soaking rice grains and boiling rice till the water becomes starchy and viscous and cooled down. The rice starch was isolated from boiled rice by the alkaline treatment method as stated by Bae et al., (2018). NaOH was added to rice water to make 0.05M of final concentration. The solution was kept overnight and passed through sieve filters; the process was repeated multiple times and solution was centrifuged at 5000 rpm for 5 minutes to obtain starch precipitate. The starch was collected and washed and neutralized with deionized water. The Rice starch collected was then air dried in hot air oven at 40°C for 48 hours. The dried rice starch was powdered and pass through standard sieve filters and stored for ethanol fermentation studies.

2.8 Soluble starch and rice starch Hydrolysis with commercial enzyme

The samples were diluted and made into 1% starch solutions; The commercial a amylase enzyme was obtained from Himedia. 10ml of 1% starch samples of soluble starch and rice starch and added with 1ml of commercial enzyme solution and incubated for 42°C for 1 hour and boiled for 5 minutes to perform enzyme inactivation. The solutions were cooled and made up to known volume. Blank, glucose standards upto 0.2 to 1.0mg/ml glucose, and samples were added with 1ml of DNS reagent and heated for 10 minutes. The samples were cooled and absorbance was measure at 540nm using spectrophotometer to assess the hydrolysed sugars in samples.

2.9 Starch analysis by Iodine colorimetric method

The samples for starch analysis were carried out by Iodine colorimetric method, The samples were extracted ad standardized iodine was added, the absorbance was measured at 620nm for starch estimation. The amount of starch was estimated by standard starch curve as reported by Sheng et al., (2022).

2.10 Separate Saccharification and Fermentation

Soluble starch and rice starch were taken at 2% and 10% level in 100mM citrate buffer, sterilized and 1ml of 300IU Amylase (Himedia) was added and incubated for 6hrs at 42°C. To 100ml hydrolysate 10ml of Amylolytic Yeast precipitate was added and measured at 42°C for 72 hrs. After fermentation reducing sugars left and ethanol produced were estimated.

2.11 Fermentation efficiency studies

The ethanol formed with respect 1gram of glucose consumed was noted as practical yield and divided by standard theoretical yield to obtain the ethanol fermentation efficiency

Fermentation efficiency (%) = Practical yield (amount of ethanol obtained from conversion of 1g of glucose) /Theoretical yield (maximum amount of ethanol that can be produced from 1g of glucose) ×100

2.12 Simultaneous Saccharification and Fermentation of Soluble starch and Rice starch

The Simultaneous Saccharification and Fermentation of 2 different starch samples was carried out in 2 different concentration levels. 2 and 10 grams of samples were suspended in 80ml of 100mM citrate buffer and 10ml of

amylolytic thermotolerant yeast pellet was added to carry out fermentation. The fermentation media was incubated at 42°C for 72 hours. The anaerobic fermentation was carried out and left out glucose was calculated by DNS method.

2.13 Ethanol estimation

The samples were diluted in distilled water the sample was passed through 0.45µm filter and analysed ethanol by Gas chromatography.

2.14 Statistical analysis

The experiments were repeated thrice in triplicates (n=9) and values with standard deviation are presented in this study.

3. Results

3.1 Sampling Screening for Amylolytic and thermotolerant yeast

The enriched broth was spread on modified media plates and incubated at different ranges of temperatures, The highest thermotolerance from enriched broth samples was observed at 42°C and selected for further amylolytic activity.

The ringer's iodine test on spread thermotolerant sample was done by pouring ringers iodine over spread plates and hydrolytic zone was observed. The isolate with highest zone of hydrolysis was subculture and confirmed to be amylolytic and thermotolerant.

3.2 Colony Morphology and Microscopic characterization

Creamy colonies with smooth surface and buttery dry texture with convex colonies of round shape and sweet odour was observed.

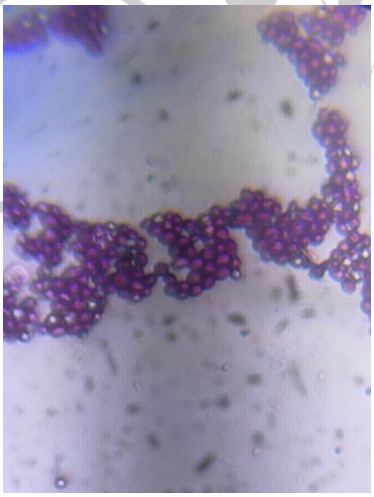

Figure 1: Microscopic observation of Amylolytic Thermotolerant *Candida*.

Microscopic observation revealed that the colonies are chain like oval colonies with 3-6µm in length with multilateral budding unlike common yeast, branching pseudo hyphae was observed. The microscopic observations revealed that the isolate belongs to *Candida* genus. Further biochemical tests were performed to identify the species of isolate.

3.3 Biochemical Characterization studies

The biochemical parameters were assessed to identify the species of isolate. The biochemical tests of urease test and sugar fermentation test are as follows:

Sugar fermentation test revealed that the isolate is positive for glucose, galactose, maltose, sucrose, trehalose and negative for xylose, arabinose, mannitol and Lactose.

Table 1: Biochemical characteristics of Candida isolate

Carbohydrate	Assimilation result (+ or positive / - or negative)
Glucose	+
Sucrose	+
Lactose	-
Maltose	+
Galactose	+
Trehalose	+
Starch	+
Xylose	+
Arabinose	-
Cellobiose	-
Sorbitol	+
Inositol	-
Erythritol	-
Glycerol	+

The biochemical characteristic studies confirm as per above sugar assimilation test profile it is confirmed as *Candida tropicalis*. The isolate is subcultured and stored at 4°C.

3.4 Disc diffusion assay and resistance profile of *C. tropicalis*

The drug susceptibility of *C. tropicalis* is confirmed by Mueller Hinton disc diffusion assay. The antibiotic susceptibility profile of various antibiotics is as follows

Table 2: Zone of inhibition by different bacteria on *C. tropicalis*

Antibiotic	Zone of Inhibition (mm)
Fluconazole	1 ±0.019
Voriconazole	3 ±0.021
Itraconazole	3 ±0.42
Posaconazole	4 ±0.24
Caspofungin	11 ±0.14
Micafungin	11 ±0.12
Amphotericin B	31 ±0.19

The isolate was found to be highly resistant against azole drugs i.e., Fluconazole, Voriconazole, Itraconazole and Posaconazole, moderate resistance was observed for Echinocandin drug class consisting Caspofungin and Micafungin. The isolate was highly susceptible to Amphotericin B.

3.5 Hydrolysis of starch samples by commercial enzyme and glucose estimation DNS method

The starch samples were hydrolysed by commercial amylase enzyme and glucose estimation was also performed.

Table 3: Hydrolysis efficiency and glucose yield of starch samples by commercial enzyme.

Starch Samples (per gram)	Starch hydrolysed	Glucose per starch hydrolysed	Hydrolysis efficiency
Soluble starch	0.71 ± 0.011 grams	0.79 ± 0.098 g	71 ±0.98 %
Rice starch	0.70 ± 0.006 grams	0.77 ± 0.044 g	70 ± 1.22 %

The treatment of starch with commercial amylase showed 71 ±0.98 % and 70 ± 1.22 % of hydrolysis efficiency for Soluble starch and Rice starch indicating commercial amylase enzyme had a decent activity of hydrolysis.

3.5 Separate Saccharification and Fermentation efficiency of Soluble starch and Rice starch

	Soluble starch (2 grams)	Soluble starch (10 grams)	Rice starch (2 grams)	Rice starch (10 grams)
Total starch before Hydrolysis	2 ± 0.03 grams	10 ± 0.015 grams	2 ± 0.14 grams	10± 0.14 grams
Starch hydrolysed	1.401± 0.014 g	7.041± 0.12 g	1.41±0.019g	7.02±1.32g
Hydrolysis efficiency (%)	70.2± 0.9%	70.4± 1.28%	70.3±1.15%	70.2±1.1%
Glucose produced	1.560± 0.29g	7.821± 0.12 g	1.562±0.027 g	7.799±0.13 g
Glucose consumed	1.558±0.02g	7.819±0.95g	1.560±0.02g	7.797±0.11g
Glucose left	0.002±0.00001g	0.003±0.00001g	0.002±0.00001g	0.002±0.00001g
Ethanol produced	0.711±0.011g	3.579±0.05g	0.702±0.011g	3.533±0.055g
Ethanol fermentation efficiency	89.3±1.1%	89.6±1.2%	88.1±1.1%	88.7±1.3%

The separate saccharification and fermentation was performed with soluble starch and rice starch as substrate. The hydrolysis was observed to 70.2± 0.9% and 70.2±1.1% for soluble starch and rice starch with 89.3±1.1% and 88.7±1.3% of ethanol fermentation efficiency for soluble starch and rice starch in 10 grams level.

3.6 Simultaneous Saccharification and Fermentation efficiency of Soluble starch and Rice starch

The Simultaneous Saccharification and Fermentation studies were done on 2 and 10 grams level and large-scale fermentation at 10 grams was also performed to assess the bioethanol production from soluble starch and rice starch samples.

Table 4: The Simultaneous Saccharification and Fermentation and fermentation efficiency studies.

	Soluble starch (2 grams)	Soluble starch (10 grams)	Rice starch (2 grams)	Rice starch (10 grams)
Total starch before Hydrolysis	2 ± 0.02 grams	10 ± 0.02 grams	2± 0.14 grams	10± 0.14 grams
Starch hydrolysed	1.742± 0.009g	8.711±0.11g	1.705±0.017g	8.521±0.012g
Hydrolysis efficiency (%)	87.2±0.4%	87.1±0.2%	85.3±0.3%	85.2±0.9%
Glucose produced	1.938±0.11g	9.677±0.15g	1.895±0.035g	9.465±0.25g
Glucose consumed	1.935±0.01g	9.675±0.095g	1.892±0.02gg	9.462±0.014g
Glucose left	0.002±0.00001g	0.002±0.000009g	0.002±0.00001g	0.003±0.000008g
Ethanol produced	0.869±0.011g	4.267±0.06g	0.825±0.014g	4.16±0.064g
Ethanol fermentation efficiency	86.3±1%	87.29±0.9%	85.2±0.9%	86±0.9%

Fermentation studies of isolate yielded grams 4.267±0.06g and 4.16±0.064g grams of ethanol for 9.677±0.15g and 9.465±0.25g grams of glucose released from starch hydrolysis of soluble starch and rice starch at 10gram level. The ethanol fermentation efficiency is observed to 87.29±0.9% and 86±0.9%. Thermotolerant amylase enzyme from yeast isolate showed 87.1±0.2% and 85.2±0.9% hydrolysis of starch substrate more compared to hydrolysis activity of commercial amylase enzyme. The isolate is concluded to be an efficient ethanol produced and consists of potential hydrolysing activity.

4. Discussion

In the present study the isolated thermotolerant amylolytic yeast with efficient hydrolysis and ethanol fermentation capacity. Voronovsky et al., (2009) developed thermotolerant and

amylolytic strains of yeast *Hansenula polymorpha* for alcoholic fermentation. The samples for isolation of *C. tropicalis* in present strain were taken from bakery samples and soil from cooking area with temperature tolerant upto 42°C, Miah et al., (2022) also reported adaptability of strain up to 42°C, Samples were drawn from warm and places with high starch content for potential encounter of yeast strains with thermotolerant amylolytic activity. The similar approach for sample collection can also be seen from the studies of Fossi et al., (2009) by collecting thermostable amylase producing yeast from starchy and warm waste soil samples. The above observations concludes that starchy waste samples with warm climatic conditions yield better chances for isolation of thermotolerant yeasts with amylolytic activity.

The isolation of rice starch from boiled rice carried out was by alkaline treatment method. This method was also practiced in the studies by Wang et al., (2022), Correia et al., (2021) and Bhatt et al., (2023)

Microscopic and biochemical tests revealed that the isolated organism is *C. tropicalis,* microscopic observations of *C. tropicalis* stated that the chain like oval colonies with 3-6µm in length with multilateral budding unlike common yeast, branching pseudo hyphae. Sugar assimilation test was also done to confirm *C. tropicalis*. These morphological and biochemical observations of *C. tropicalis* were also performed by Zuza-Alves et al., (2017), Osumi et al., 1975 and Satala et al., (2022) and confirmed similar findings as per the present study.

The resistance profile of *C. tropicalis* was studied and concluded that the strain is highly resistant to azole drugs and Echinocandin resistance was less compared to Azole group of drugs, the strain was found to be susceptible to Amphotericin B. Many studies have reported the development and prevalence of drug resistant, the resistance to azole group of drugs was most reported, Lima et al., (2022) reported antifungal resistance of *C. tropicalis* highlighting the resistance of azole group drugs. Dos Santos et al., (2023), Paul et al., (2022) and Chen et al., (2021) have reported high azole drug resistance of *Candida tropicalis* species. Khalifa et al., (2022) reported the Azole and echinocandin resistance of *C. tropicalis* in Japan.

Simultaneous Saccharification and Fermentation is advantageous over Separate Saccharification and Fermentation due to simultaneous enzymatic hydrolysis and fermentation achieved by one organism without relying on commercial enzyme and other organisms. In this present study Simultaneous Saccharification and Fermentation yielded more hydrolysis efficiency compared to hydrolysis by commercial enzyme. The isolate reported 87.29±0.9% and 86±0.9% of ethanol fermentation efficiency for soluble starch and rice starch. Oberioi et al., (2010) reported 77% of ethanol efficiency by *C. tropicalis* via fermentation of rice straw, Simultaneous Saccharification and Fermentation was concluded more economically conventional methods and was reported by Pinaki et al., (2015) and Ogren et al., (2007) concluded Simultaneous saccharification and

fermentation to be cheap and more efficient than Separate hydrolysis and fermentation, these findings were also concluded in the present study.

5. Conclusion

The study concluded the isolation of *C. tropicalis* with amylolytic and thermotolerant activity growing at 42°C. Soluble starch and rice starch was used as the substrate for hydrolysis and fermentation studies. Simultaneous saccharification and fermentation is proven to have more hydrolysis efficiency and amount of ethanol produced compared to separate saccharification and fermentation. This concludes that thermotolerant *C. tropicalis* can hydrolyse and produce bioethanol from industrial and agricultural starch waste with highly hydrolysing and ethanol fermentation capacity.

6. Reference

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