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Production of a thermostable amylase by yeast strainisolated from sahari-ansoils cultivated in Soft cheese whey

S. Toumi, A. Tifrit, D. Hadjazi, Z. Chama and B. Abbouni

Laboratory for Molecular Microbiology Proteomics and Health, Department of Biology, Faculty of Natural Sciences and Life, University of Djillali Liabès of SidiBel Abbes, Algeria

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ABSTRACT

Eleven yeasts strains were isolated from Saharian soils Bechar and Ouargla(Algeria), screened for their ability to produce amylases. Phenotypic characterization of these amylases producing strains revealed the prominence of as-comycetous yeasts. A selected strain, Schwanniomyces sp, showed the highest amylase activity on starch agar plate. This work investigated the capacity of the yeast LB_3 of producing amylolytic enzymes through fermentation in cul-ture medium, based on soft cheese whey to 100% deproteinized and supplemented with soluble starch to 1%.The yeast LB_3 showed the highest peaks of enzymatic production at 56 h, where 4796 U/ml of amylase were obtained at 30°C and pH 5. The optimum enzyme activity was displayed at 60°C and pH 6. About 91%and 85%of the activity retained after heating the crude enzyme solution at 60°C and 70°C respectively for 2 h and enzyme was completely inactivated when heated at 120°C for 190min. The extracellular amylase was found to be thermostable and active at wide range of pH. Of the carbon sources, Wheat starch was found to be very carbon source for enzyme production. Yeast extract and ammonium nitrate (0.2%) as nitrogen sources gave higher yield compared to other nitrogen sources. The enzyme was strongly inhibited by Zn^{2+}, Fe^{2+},Hg^{2+}, Cu^{2+} andAg^{+}but stimulated by Ca^{2+} and Mn^{2+}. En-zyme was very sensitive to SDS (2%)and inhibited by 5 mM EDTA.

Key words: Yeasts, Saharian soil, *Schwanniomyces*, amylase, Cheese whey, Thermostability.

INTRODUCTION

Amylases are among the most important enzymes used in biotechnology, particularly in process involving starch hydrolysis. Though amylases originate from different sources (plants, animals and micro organisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermostability [1].Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins, and progressively smaller polymers composed of glucose units [2]. Thermostable enzymes from micro-organisms have found a number of commercial applications because of their overall inherent stability [3].The most widely used thermostable enzymes are the amylases in the starch industry [4-6].The present study is aimed to isolate yeasts strains producing thermo-stable amylase from Algerian soils (Sahara), These strains are also selected by their ability to ferment the cheese whey, which is a by-product of cheese production, only half of the cheese whey production is used for different pur-poses, including production of biogas, ethanol, and single-cell protein, whereas the other half is discarded as effluent [7]. Thus, cheese whey causes a major environmental problem due to its high biological oxygen demand and the high chemical oxygen demand, primarily as a result of its high lactose content [8]. Indeed, in Algeria, the overall amount of whey, jetty daily, is 6000 liters per production unit [9].The inadequate destination of this residue can cause large passive environmental issues for the industries. One of the destinations of this residue can be the feeding of domestic animals, when associated to other nutrients. In this way they become a low-cost substrate for yeasts growth and the obtention of enzymes of biotechnological interest. The present work evaluated the capacity of yeasts strains of producing the amylolytic enzymes through fermentation in medium composed of whey cheese , and to

characterize the strains productivity and the enzyme produced, particulary their behaviour toward temperature,pH and culture conditions .

MATERIALS AND METHODS

Sample collection

Soil samples were collected from different areas in the Sahara of Algeria: Bechar (South-eastern of Algeria), Ouargla (Hassi Messaoud).The samples were collected in a sterile container and brought to the laboratory for further processing.

Isolation and partial characterization of strains

The samples were serially diluted up 10^{-6}dilutions by the serial dilution method. Yeasts isolations were performed on Yeast Malt Agar (YMA) media plate containing antibiotic gentamicin to ovoid bacterial contamination. Inoculated plates were incubated at 30°C for 24 to 48 hours. The colonies were purified several times on YM agar plate. Morphological characteristics of yeasts were identified according [10, 11].

Amylase screening

Amylases producing yeasts were screened on Amylase Activity Medium (AAM) [soluble starch 5g/l; peptone 5g/l; yeast extract 5g/l; $MgSO_4.7H_2O$.5g/l; $FeSO_4.7H_2O$ 0.01g/l; NaCl 0.01g/l; agar 15g/l] plates. Incubation at 30°C was carried out for 3 days, after which the plates were stained with lugol solution. The colonies forming the largest halo zone were selected for further investigation [12].

Production of amylase

Biochemical composition of cheese whey

The cheese whey was collected at “GIPLAIT” production unit of region of Sidi Bel Abbes. This product comes from the manufacture of cheese "Camembert". The samples were collected in plastic bottles, and preserved by freezing at -20°C.

The content in ashes is determined by incineration of cheese whey at 550°C during 5 hours [13].The total sugars were determined by the method of Dubois [14]. The total nitrogen is determined by the method of Kjeldhal [13, 15]. The mineral salts are determined according to the methods advocated by [16]. Concentration of the lactic acid was determined by acidity titration with NaOH [16].

Growth medium

The basal medium was prepared at different concentrations ranging from 10 % to 100% in deproteinized cheese whey, in order to determine the optimal concentration for the production of amylase.Furthermore, the basal medium was supplemented with inductor substrate (starch at 1%) and then adjusted to pH 5[17].

Assay of enzyme activity

Extracellular amylases were produced in submerged fermentation; this production was carried out in 250 ml Erlenmeyer flasks containing 50 ml of liquid medium for enzyme production. The media were then incubated with a cell suspension at a rate of $2.6x10^6$ cells /ml at 30°C in an orbital shaker set at 180 rpm for 72 h. Three replicate fermentations were carried out for each culture medium. The enzyme assay was performed according to the method by Rick and Stegbauer [18]. The enzyme extract (0.5ml) was transferred to a test tube containing 0.5 ml of 1 % soluble starch solution. The mixture was incubated at 60°C for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The absorbance was determined at 546 nm using a spectrophotometer and converted to mg of maltose from the standard. One unit is equivalent to that amount of enzyme, which catalyze the hydrolysis of soluble starch into 1.0 mg maltose hydrate per minute under standard assay conditions.

Assay of protein concentration

The protein concentration was determined by the Lowry’s method [19] by usingbovine serum albumin as the standard.

Optimization of amylase production

Effect of pH and Temperature

The enzyme activity was investigated by the study of the effect of pH on extracellular amylase production. For this, the medium was prepared with varying the pH values ranging from 2.5 to 8.5. Optimum temperature for enzyme activity was determined by conducting the assay at different temperature ranging from 20 to 90 °C.

Thermo stability of amylase

The thermal stability of the enzyme was determined by incubating the enzyme fraction at various temperatures (60, 70, 80, 90,100,110 and 120°C) for 190 min, at 15 min intervals [20].

Effects of different carbon sources

The effects of different carbon sources (Glucose, Glycogen, Corn starch, Potato starch, Wheat starch, Soluble starch, Maltose, Xylose, Isomaltose, pullulan, Amylopectin) on amylase production by the yeast were examined at a concentration of 1%, keeping constant the rest of the media composition.

Effects of different nitrogen sources

In order to investigate the effects of different nitrogen sources on amylase production, was replaced with different organic (yeast extract, casein, beef extract and peptone,) and inorganic (ammoniumnitrate, ammonium sulphate) compounds as nitrogen source at a concentration of 0.2% (w/v).

Effect of metal ions, SDS and EDTA

The influence of various metal ions (1 and 5µmM) was determined by adding Ca^{2+}, Zn^{2+}, Fe^{2+},Fe^{3+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Na^{+}, K^{+} and by monitoring the residual activity. Amylase stability was studied by preincubating the amylolytic preparation for 30 min at room temperature and pH 6. Activity in the absence of any additives was taken as 100%. The effect of surfactant (SDS) and enzyme inhibitor ethylene-diamine tetra acetic acid were also studied.

RESULTS AND DISCUSSION

Isolation, selection and partial characterization of strains

Isolation and selection of suitable organism is very necessary for maximum production of Amylases. Primary screening was carried out by starch hydrolysis method. Eleven yeasts strains presented significant amylolytic activity (Figure 1), Those that amylolytic power was upper or equal to 18 mm on AAM agar plate after 3 days incubation at 30°C were considered as amylases overproducing strains, and were identified on the basis of their morphological, physiological and biochemical properties according to Lodder and Kreger keys[10, 11].Ovoid shape was prominent among yeasts while rectangular was minor. The cell size varied from 2 To 4 µm in diameter. The asexual reproduction mod, unilateral or multilateral bundings cells were observed after 19 h of culture at 30°C for all strains , pseudomycelium were observed for strains LB_3, LB_4 and LH_9. None mycelium was observed for strains LH_5 and LH_7. The behaviour of yeasts after cultivation on sporulation medium [21]was essential for their distinction in two main groups: *Ascomycete* groups that were able to give spores: this group was represented by strains LB_3, LH_5 and LH_9; their vegetative cells were transformed directly into asci after 4 days of incubation at 30°C in sporulation medium. The asci contained 1 to 6 spheroid ascospores. The second group found was unable to release spore after 7 days of incubation at the same temperature, this group was classified as *Deuteromycetes*, and represented by strains LB_4 and LH_7.Strains LB_3, LB_4 and LH_7 have negative reaction to nitrate reduction test while fermentation of sugars (Glucose, Maltose, Saccharose) were all positive, Strains LH_5 and LH_9 have positive reaction to nitrate reduction test. Almost strains were not able to fermentxylose; however, they were all positive for starch hydrolysis. All strains were able to growth at 37°C. According to their morphological and biochemical profile, the strains LB_3, LB_4, LH_5, LH_7, and LH_9 were respectively belong to the genus *Schwanniomyces*, *Candida*, *Lipomyces*, *Cryptococcus* and *Saccharomyces.*
The stain LB_3 showing the highest amylolytic halo was selected for further analysis (Figure 2).

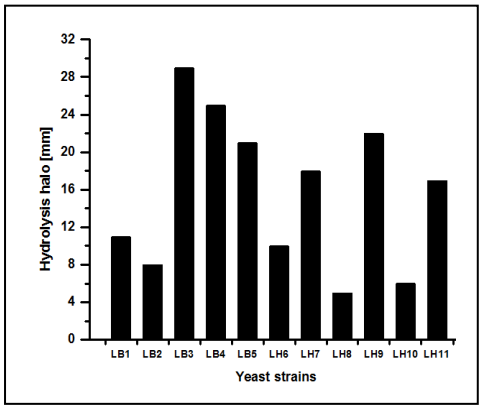

Figure1:Amylase activity of the isolated yeasts strains on in cheese whey culture medium containing soluble starch, incubated at 30°C for 72 hours L: Yeast; Band H: Geographic origin [Bechar, Hassi Messaoud]

Figure2:Illustration of the LB_3producing amylase yeasts strain, on cheese whey culture medium containing soluble starch, incubated at temperature of 30°C for 36hours

Production of amylase

Study of the chemical composition of whey

Analysis of the results was showed that the soft cheese whey was rich on total sugars 67g/l, e30°C for 36hspecially lactose (47.87g/l). Its lactose wealth was an important source of carbon and energy for the growth of many microorganisms with the system b-galactosidase [22-23].This high lactose content was approached the values of 40-57g/l[24].

Dry matter obtained was 61g/l; our finding is in a good agreement with [24].The protein content of 6.02g/l was a value slightly less than that reported by [24].This difference in protein content was may be due to the cheese manufacturing process [25].The lactic acid content of 2.8 g/l was similar to that reported inlitherature [26].The mineral was rich in chlorine (3.4g/l) and calcium (0.35g/l), activating factors of α-amylase [27, 28].However, the pH value 5.97 was slightly less than that specified by literature (pH 6.5- 6.7) [29].This difference may be due to transportation and storage conditions of whey.The fat was showed a value of 0.72 g/l. This finding is in agreement with the studies reported earlier[24, 30].The total nitrogen matter obtained was 1.2g/l. A similar result was also found by[24, 26].In face of the results obtained, it is verified that cheese whey can be used as a fermentation medium for the production of amylases.

The obtained results were indicated that the production of amylase was proportional to the concentration of whey, at a concentration of 100%, the amylase activity was maximal4591 U/ml (Table 1). The strain*Schwanniomyces sp* was required a fermentation medium composed of 100% by deproteinized cheese whey and supplemented with starch to 1% (w/v).

Table 1: Effect of cheese whey concentration on amylase production

Concentration of whey (%)	Amylase activity (U/ml)
10	950
20	1010
30	1607
40	1924
50	2010
60	2540
70	2986
80	3676
90	4150
100	4591

Production of amylase by *Schwanniomyces sp*

The obtained results allowed the construction of a curve representing the kinetics of the behavior of the enzyme during the whole fermentation. The following variables were considered: Carbohydrates, Biomass and amylase activity. A linear relationship was found between enzyme synthesis and Biomass i.e. maximum amylase production occurred when the biomass reached to maximum. The production of amylase by *Schwanniomyces sp* was started at the beginning of the exponential growth. The measurement of biomass and amylase activity in cheese whey fermented broth

during 96 h showed that both enzyme activity and biomass increased exponentially with the time of incubation up to 56 h. The activity was 4796U/ml at that time, afterwards there was a drastically increase in activity (Figure 3).It might be due to the accumulation of other by- products in the medium with high level of protease activities and depletion of the nutrients [31].It was found that α-amylase production was maximum when starch was used as the carbon source[32-38].

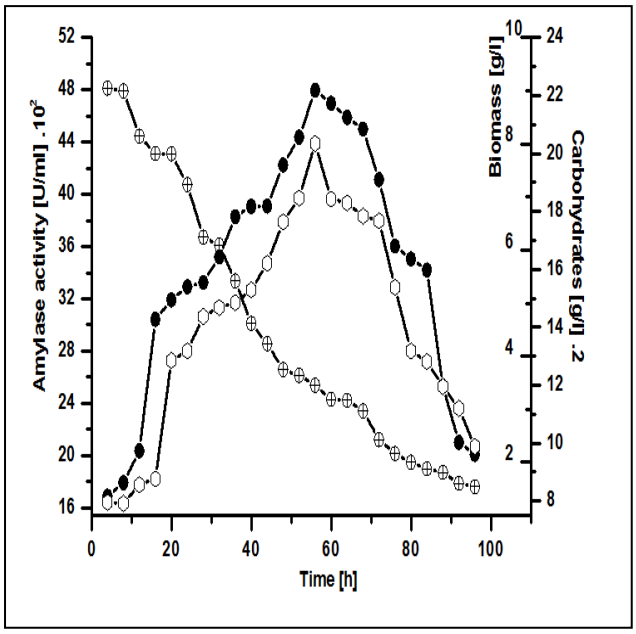

Figure 3: Time course of amylase production[ ], carbohydrates degradation [ ] and biomass [ ] by *Schwanniomyces sp* in cheese whey culture medium containing soluble starch

Evolution of pH and proteins during the fermentation

Among physical parameters, pH of the growth medium plays an important role in enzyme secretion. The pH range observed during the growth of microbes also effects product stability in the medium[39].In this work, the initial pH value was 5 and has changed during the process of fermentation from 5-6.62 in the first 28 hours.This increase in pH is may be due to the protein degradation that release amino acids then hydrolyzed into ammoniac by deamination[31]. ThepH increase is also explained by the metabolism of carbohydrates which releases CO_2.Reaching 5.02 at 64 h of fermentation and keeping close to 4.6 until the end of the process (Figure 4).pH resumes its decline due to the formation of organic acid from carbohydrates[40].Our study showed a slight increase during the growth phase explained by the synthesis of enzyme in particular hydrolases[41].

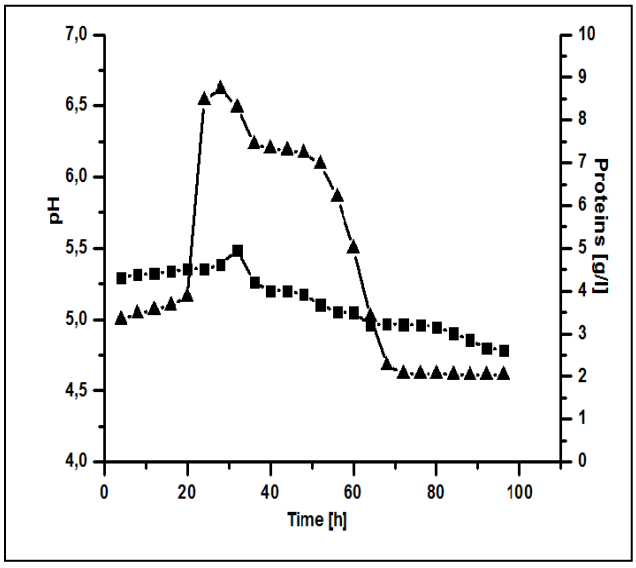

Figure 4: Kinetic profile of proteins [ ] and pH [ ].

Optimization of amylase production

Effect of pH and temperature on extra cellular amylase production. The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of alpha amylase[42].In order to investigate the effect of pH on enzyme production, the initial pH of the medium was adjusted from 2.5 to 8.5. The amylase was active in the pH range 2.5-5.5 and maximum enzyme yield was observed at pH 6(Figure 5). Further, increase in pH resulted decrease in the production of amylase by the yeast culture. At neutral pH, the results were moderate and at acidic pH the enzyme activity was extremely low. It might be due to the fact that the enzyme was inactive in the acidic medium[43].Each enzyme has an optimum pH at which the activity is maximum. The optimum pH 6 for the amylase of Schwanniomyces sp was close to 6.3 for the yeast *Schwanniomycesalluvius*[44].and 5.6 for the yeast *Filobasidiumcapsuligenum*[45].Temperature and pH are the most important factors, which markedly influence enzyme activity.The effect of temperature on enzyme activity was assayed at different temperatures ranging from 20-85°C. The results showed that enzyme activity was increased with temperature and it showed highest activity at temperature 60°C (Figure 6).Further increase in temperature resulted in decrease in the activity of amylase. Similar findings have been reported on *Lipomycesstarkeyi*[46], and *Aureobasidiumpulluians*[47].

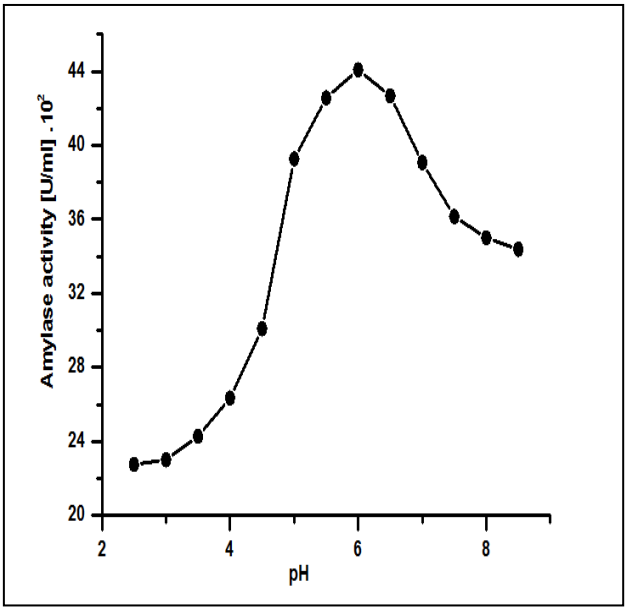

Figure 5: Effect of pH on the production of extracellular amylase by *Schwanniomyces sp*

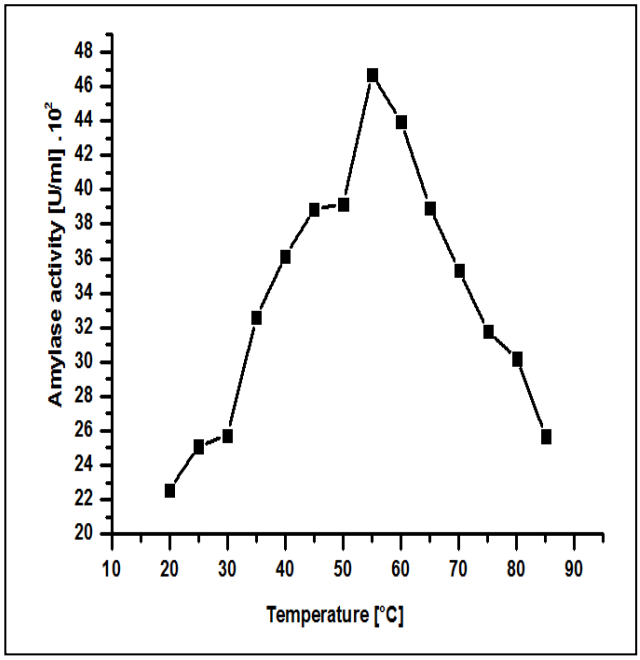

Figure 5: Effect of temperature on the extracellular amylase production of by *Schwanniomyces sp*

Thermo stability of amylase

Stability of the enzyme is of great importance for the economy of their industrial application. In case of thermostability, the enzyme was pre incubated at different temperatures for 190 min and then enzyme was assayed. The results showed that the enzyme activity was retained 91%, 85% after heating at 60°C and 70°C respectively for 2hs (Figure 7). After this time the activity was decreased and enzyme was completely inactivated when heated at 120°C for more than 190 min. The temperature stability indicate a general decrease in stability of the enzyme with time (10-190min) at temperatures 80-90-100-110 and 120°C respectively as shown by decreased enzyme activity. It showed the highest stability at 60°C and least stability at 120°C.Temperature is one of the factors that induce such changes on cell membrane and cell wall. The high temperature inactivation may be due to incorrect conformation due to hydrolysis of peptide chain, destruction of amino acid, or aggregation [48].Further, the thermostability of this enzyme greatly exceeds the reported values by [49], where *Cryptomyces sp* yeast retain only 20% and 10% of its activity after heat treatment of 30 min at 80°C and 90°C.It also greatly exceeds that of the yeast *Aureobasidium pullulans* that which denatures completely at 70 ° C for 30 min [47].Our results are similar with other studies in case of-*Schwanniomycesalluvius*[50].The behavior of yeast strain LB_3, particularly, the thermostability of its amylase, bring out some hypothetic considerations, taking into account the natural medium where the yeast strain have been isolated [51]. On the other hand, gene mutation could be another reason explaining this particular behaviour of yeast strain LB_3. Thus, the results concluded that the crude enzyme is moderately temperature stable. It is therefore worthwhile to consider means to stabilize the enzyme under storage conditions.

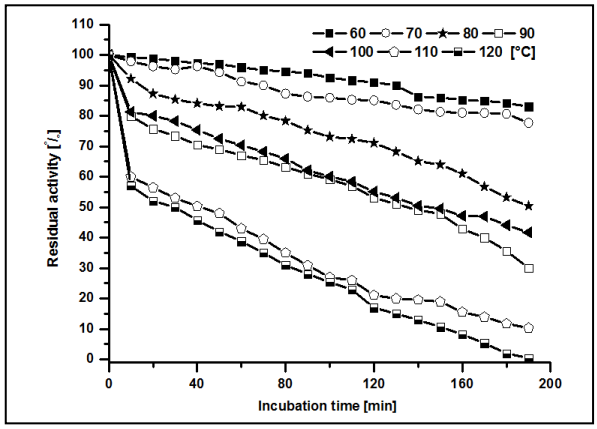

Figure 7: Effect of temperature on enzyme stability.

Effect of carbon sources on extra amylase production

Amylase production in yeast is effected by the nature of the carbon sources. The results indicated that the biosynthesis of the enzyme took place not only in the presence of starch but also with other carbon sources. Our study showed that the enzyme hydrolyzed all starches used as carbon sources in the basal media with maximal activity on wheat starch, followed by potato (Table 2). Earlier studies reported that, complex substrates induce higher amylase production [38].On the other hand, glucose was a very poor substrats. Isomaltose and pullulan were not hydrolysed at all. The carbon sources are important factors affecting cell growth and product formation of microorganisms. It has been suggested that the induction of α-amylase is mediated by starch or starch breakdown products such as maltose [52-56].The carbon sources may have either repressing or inducing effects on enzyme productions. Glucoamylase, amylase, and alpha-glycosidase are all upregulated by starch and down-regulated by glucose[57].

Table 2: Effect of different carbon sources on extracellular amylase production

Carbon sources	Amylase activity (U/ml)
Glucose	06.04
Glycogen	13.6
Corn starch	36.8
Potato starch	39.9
Wheat starch	42.8
Soluble starch	34
Maltose	n.d
Xylose	28.6
Isomaltose	00
Pullulan	00
Amylopectin	19

**n. d : not detectable.*

**Results are mean values of three replicates.*

Effects of various nitrogen sources on amylase production

In general, both organic and inorganic nitrogen sources were used efficiently in the growth medium for the biosynthesis of amylase. In present study, yeast extract, casein, beef extract and peptone showed stimulating effects on amylase synthesis (22.8U/ml, 20.6U/ml, 18.2U/ml, 14.9U/ml) respectively. It has been reported that yeast extract also served as good organic nitrogen source for α-amylase synthesis from *Bacillus amyloliquefaciens*[58-59]. Earlier studies reported that, optimum activity at 0.02gm of peptone content on agro industrial waste by *AspergillusSp*[60].Krishna and Chandrasekaran reported production of α-amylase by *Bacillus subtilis* on banana fruit stalk and got optimum activity at 0.5gm peptone content[61].Similarly, casein was reported to be a good nitrogen source for α-amylase production from *B. subtilis IP 5832*[37].

It was observed thatinorganic nitrogen sources gave comparatively higher yields than organic nitrogen sources. In our study, the enzyme production was increased when ammoniumnitrate and ammonium sulphate used as inorganic nitrogen sources in the culturemedia (58U/ml, 52.67U/ml) respectively. According to[62] ammonium salts were stimulators of *B. subtilis* amylase synthesis.Our findings are in a good agreement with the findings of these studies. It has also been reported that, ammonium nitrate and sodium nitrate were the best nitrogen sources for maximum amylase production [63, 64].

Effect of metal Ions, surfactants, and enzyme Inhibitors

The influence of metal ions on the activity of extracellular amylase was also studied. It was found thatamylase activity was greatly inhibited by 5 mM of Zn^{2+}, Fe^{2+},Hg^{2+}, $Cu^{2+}$and Ag^{+}. Whereas 5 mM of Ca^{2+} and Mn^{2+} showed stimulating effect of 181% and 172% increase, respectively. However, K^{+} and Na^{+} have pronounced effect in decreasing the enzyme activity(Table3). These results suggest that calcium is needed for the optimum activity and stability of the enzyme. A similar result was also found in case of Pichia *burtonii*, *α*-amylase was activated by Ca^{+2} (135%) [65].These authors proposed that the activation of enzyme by calcium ions probably happened during the purification procedure when the enzyme lost activity. The *Bacillus halodurans* 38C-21 *α*-amylase also had its activity increased in presence of Ca^{2+} [66].

Table 3:Effectof metal Ions and EDTA on the amylase of Schwanniomyces *sp*

Reagents	Relative activity (%)	
	1mM	5mM
$HgCl_2$	61	26
$ZnSO_4$	62	29
$CuSO_4$	57.9	23
$MnSO_4$	126	172
$MgSO_4$	96	84
$CaCl_2$	129	181
$CdCl_2$	87	64
$FeSO_4$	58	28
Fe^{3+}	74	57.6
NaCl	90	82.7
KCl	91	72
Ag^{+}	60	25
EDTA	42	35

The presence of Mn^{2+} ions had prominent activating effect (147%) on amy1ase activity. It was observed that Mn^{2+} had no effect on α-amylase activity [35, 67]. However, It has been reported that production decreased from 570 U to 425 U in medium containing Mn^{2+}[68].However, Zn^{2+}, Cu^{2+}, and Hg^{2+} acted as inhibitors of amylase activity. Early studies showed that the effect of Zn^{2+} varied between amylases. Another study tested the effect of Zn^{2+} and they found that amylase production decreased from 570 U to 415 U[68]. The results are also confirmed by[69], who stated that the presence of Zn^{2+} had a potent inhibitory effect on the amylases from *Schwanniomyces alluvius* and *Bacillus cereus NY 14*.It has also been reported that Zn^{2+} strongly inhibited the enzymatic activity (91%) of alkaliphilic-*Bacillus sp*[70]. As for the thermostable α-amylase from a thermophilic *Bacillus* 46% [35].and 13% inhibition were reported, suggesting that the inhibition with Zn^{2+} determines the thermostability of the enzyme [71].It was observed that Zn^{2+} showed 37% inhibition on enzyme production from *Bacillus sp*[1].The inhibition by Hg^{2+} may indicate the importance of indole group of amino acid residues in enzyme function [32, 50]. The wild-type amylase is inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, andMg^{2+}[49], while that from *L. kononenkoae* CBS 5608 is inhibited by Ag^{+} and Cu^{2+}[72].Role of Ca^{2+} and Mg^{2+} in maintaining the stability and structure of the α-amylase is well documented [73]. Enhancement of amylase activity of Ca^{2+} ions based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acid, which resulted in stabilization as well as maintenance of enzyme conformation. In addition, calcium is known to have a role in substrate binding. It has also been documented that binding of Ca^{2+} to amylase is preferred over other cations such as Mg^{2+}[73].Enzyme activity was inhibited by 5 mM EDTA in 1h. In case of *L. kononenkoae*α-amylase, enzyme activity was not affected by EDTA [74].Increase in detergent (SDS) content

from 1% to 2% decrease relative activity from 29% to 13% after 1h of incubation. Amylase activity was very sensitive to SDS. This Anionic detergent (SDS) is significantly known to disrupt the proteins higher ordered structure.

CONCLUSION

In this research, it could be conclude that the cheese whey is very rich in lactose 47.87g/l which make it a substrate of choice for the development of high value substances andcan constitute a fermentation medium of good quality for biosynthesis and production of amylolytic enzymes, because it induced microorganisms to express the genes for the enzyme assayed. The particularity of this work is the founding of amylolytic yeast LB_3 presenting a particular character due to its high amylolytic activity4796 U/ml and heat stability of its crude amylase extract (60°C). It should also be noted that amylase producer strain with increasing enzyme activities at high temperature are promising candidates for the industrial applications and utilization of low-value agro-industrial residues as substrates should be focused upon for enzyme production, as this would reduce the cost of production and help to solve their disposal and pollution problems. For a final breakthrough to achieving commercially relevant quantities of this enzyme and will review the current state of knowledge on the molecular basis of enzyme production by yeasts in coming future prospects.

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