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Der Pharmacia Lettre, 2015, 7 (6):72-80
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Production and optimization of α-amylase by *Bacillus* strains isolated from the soils of the North West of Algeria

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ABSTRACT

The study was focused on the isolation of the amylase producing bacteria belonging to the genus Bacillus and the optimization of several parameters such as cultural conditions and the nutritional requirements implicated in the production process. For the isolation of the amylase producing bacteria, six samples of soils and water were collected from the main geographic zones in North West of Algeria such as the river of Mekerra, Lake of Sidi Mohamed Benali, wastewater treatment plant of Sidi Bel abbes, field of potato in Ain Temouchent and the factory in Maghnia, where starchy wastes are submitted to natural fermentation. Fifty four bacterial strains of the genus Bacillus were isolated and selection of strains that produced most amount of amylase was determined on the basis of two criteria: the diameter of the zone of hydrolysis in solid medium and reducing sugar (DNS method in liquid medium). Furthermore, the most efficient strain was identified by the using of the API system as B. subtilis with a yield of 1,537 U/mL. Maximum production of amylase was obtained after optimization of the following parameters: starch concentration, nitrogen source, concentration of the inoculum and metal ions effect. Productivity was increased 6 times: 11,746 ± 0, 0028 U/mL in the opt imized medium with the following composition: starch 5 g/L, gelatin 5 g/L, $CaCl_2$; 0.2 g/L and 0.5 % v/v of inoculum.

Key words: North West Algeria, α-amylase, *Bacillus sp*, Optimization, optimum activity.

INTRODUCTION

The northwest Algeria region is characterized by a multitude of landforms such as sea, mountains, plains, forest, rivers and a Mediterranean climate, which offer to this geographical location a rich biodiversity and remains unexplored microbiologically. Therefore, it is of interest to characterize the microbial population present in the soils and the water of the North West Algeria.

The α-amylase is class of enzyme (E.C.3.2.1.1), which was responsible for hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products such as glucose, maltose [1, 2].

Furthermore, amylases are used for hydrolysis of starch in food industry and for removing of starch sizing in textile industry [3, 4].

Industrial amylases productions cover about approximately 25% of the world enzyme market such as sugar, textile, paper, brewing, detergents, and the pharmaceuticals [5-10].

α-amylase has obtained from several sources, such as plants, animals and microorganisms [11]. The employed microorganisms for amylases production constitute a major advantages due their economical bulk production capacity and their easy purification [12].

Furthermore, amylases have been produced by several fungi, yeast, bacteria and actinomycetes but members of the genus *Bacillus* are heterogeneous and very versatile in their adaptability to the environmental conditions.

The great diversity within the genus of *Bacillus* species and the availability of the range of genetic tools for their manipulation hold out the promise in the future for the production of many new products [13].

Early, a partial purification of α-amylase from various *Bacillus species* such as *B. megaterium, B. subtilis* and *B. licheniformis* SPT 27 has been achieved [14, 15, 16]. The physicochemical parameters, such as the composition of the growth medium, pH of the medium, phosphate concentration, inoculums age, temperature, aeration, carbon source and nitrogen source implicated for α-amylase production has been thoroughly investigated [2]. Therefore, the objectives of the study were the isolation and the selection of the high producer α-amylase strains belonging to the genus *Bacillus* from the soils of the North West of Algeria and the optimization of the cultural conditions such as and the nutritional requirements.

MATERIALS AND METHODS

Sample collection

In order to increase the isolation of the amylase producing bacteria, six samples of soils and water were collected from the main geographic zones in North West of Algeria such as the river of Mekerra, Lake of Sidi Mohamed Benali, wastewater treatment plant of Sidi Bel abbes), field of potato in Ain Temouchent and the factory in Maghnia, where starchy wastes are submitted to natural fermentation.

For this purpose, 50 g of soils were especially collected at the places where the degradation of starchy material was remarkable and visible and a volume of 50 mL water from the above described sources of sampling. After that, the samples of soils and water were introduced in sterile polyethylene bag, transported, brought at the laboratory and kept at 4 °C.

Isolation of bacterial strains from soil sample

In order to isolate a producer of α-amylase strains belonging to the genus *Bacillus*, one gram of the soil sample was weighed and introduced into 9 mL of sterile distilled water and then subjected to heat shock 10 min at 80 °C [17] .

Serial dilution of 10^{-5} of 1 mL of the mixture was introduced into a sterile Petri dish and inoculated on starch agar plate and incubated at 37 ºC for 72 hours [18].

Different bacterial strains obtained as typical colonies were sub-cultured and thereafter purified several times on fresh starch agar plate. The isolated pure strains were screened for the production of extracellular α-amylase production using starch agar as described by [19, 20].

The pure cultures were streaked at the center of the sterile starch agar plates and the plates were incubated at 37 °C for 24 hours. After incubation, 1% iodine solution was over-layered on the agar plates and observation was made to note the substrate utilized zone around the colony [21]. The strain that formed the largest zone of hydrolysis was taken for further study. The selected α-amylase producing MK-7 strain colonies belonging to the genus *Bacillus* manifested a positive hydrolysis zone has been preliminary identified by the test of the Gram straining and the presence of spores with a positive catalase [22].

Inoculum preparation

In order to prepare fresh pre-culture, the test isolates was inoculated in nutrient broth, incubated at 37 °C on a rotary shaker for 24 hours. After that, the main culture was inoculated into sterile starch medium with a volume of 10 % of 24 hours old fresh pre-culture and incubated at 37 °C on a rotary shaker at 75 rpm for 72 hours.

The 24 hours old culture was maintained as stock cultures in starch agar slants and stored at 4 °C for regular subculturing. Whereas, the fresh over night culture was used as inoculum for further growth study and enzyme the production.The used media had the following composition (in g/L): 10 g starch, 10 g of nutrient broth, 15 g agar, served as inoculation media for all the experiments.

α-amylase activity assay

The α-amylase activity was assayed according to the protocol described by Bernfeld, [22].

A volume of 2 mL of supernatant harvested by centrifugation at 4. 000 g for 15 min from 72 hours bacterial growth culture. A volume of 0.5 mL of supernatant was added to 0.5 mL soluble starch (1% w/v) in 0.1 M phosphate buffer

(pH 6.4). The reaction mixture was incubated in a water bath at 40 °C for 30 min. A blank consisting o f 0.5 ml soluble starch (1%, w:v) in 0.1 M phosphate buffer (pH 6.4) was also incubated in a water bath at the same temperature and time with the other test tubes.

The reaction was stopped by adding 1 mL of DNS reagent in each test tube and then immersing the tubes in a boiling water bath for 5 min after which they were allowed to cool and 5 mL of distilled water was added and the reducing sugars liberated were estimated by the 3, 5 dinitrosalicyclic acid (DNS) method [19]**.** The absorbance for all the test tubes was measured at 540 nm with spectrophotometer. An enzyme unit is defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate per min at 40 °C [23].

Identification of the efficient strain

The fresh isolates were morphologically and phenotypically characterized using different biochemical tests: Gram reaction, catalase and oxidase production, indole and urease production, gelatin liquefaction, hydrolytic activities, ability to use different compounds such as various carbon and nitrogen sources. The phenotypic identification of the isolates indicated their appearance to the genus of *Bacillus*. Furthermore, the selected α-amylase producing MK-7 strain was identified at the species level by the using of API 50CHB system according to the manufacturer's instructions described by Biomerieux, France.

Optimization of the production medium and culture conditions

The selected α-amylase producing MK-7 strain was retained for the optimization of various process parameters to maximize the α-amylase yield. For this purpose, the starch concentration, the nitrogen source, the inoculum concentration and a different metal salts on α-amylase activity were investigated.

Effect of the starch amount on α-amylase activity

The appropriate amount of starch concentration on α-amylase production was studied by incubating of the optimized culture medium, inoculated with the selected α-amylase producing MK-7 strain, agitated at 75 rpm for 72 hours at 37 °C, in the presence of the following concentrati on of starch (0.5, 1, 1.5, 2, and 2.5 %). The α-amylase activity was measured by DNS method and the optimum concentration of starch was used for the optimization of further parameters.

Effect of the nitrogen source on α-amylase production

In order to explore the effect of the nitrogen source on enzyme production, the selected α-amylase producing MK-7 strain was inoculated in the optimized culture medium in the presence of several of nitrogen sources such as: urea, ammonium sulphate, gelatin, pepton and casein and incubated at 75 rpm for 72 hours at 37 °C. Amylase production was measured by DNSA method and the optimum of the nitrogen source was used for the optimization of further parameters.

The effect of the inoculum concentration

The influence of the inoculum concentration on the α-amylase production was studied by incubating of the optimized culture medium, inoculated with the selected α-amylase producing MK-7 strain, agitated at 75 rpm for 72 hours at 37 °C, in the presence of the following of the inoculums concentration (0.5, 1, 1.5, 2% w/v). The α-amylase production was measured by DNS method and the optimum of the inoculums concentration was used for the optimization of further parameters.

Effect of different metal salts on α-amylase activity

The effect of different metal salts on the α-amylase production was studied by incubating the optimized culture medium, inoculated with the selected α-amylase producing MK-7 strain, agitated at 75 rpm for 72 hours at 37 °C, in the presence of the corresponding metal salts, namely, calcium chloride, magnesium sulphate, ferric chloride, zinc sulphate, sodium chloride, potassium chloride, each at a concentration of 1-5 mM was mixed with the moistening agent individually [24]**.** After incubation, α-amylase was extracted and assayed for its activity.

RESULTS

Six samples of soils and water were collected from the main geographic zones in North West of Algeria such as the river of Mekerra, Lake of Sidi Mohamed Benali, wastewater treatment plant of Sidi Bel abbes), field of potato in Ain Temouchent and the factory in Maghnia, where starchy wastes are submitted to natural fermentation.

Totally, fifty four bacterial isolates belonging to *Bacillus* species were screened for α-amylase production on starch agar medium. Colonies with highest clear zone on the plate agar medium by the addition of 1% iodine solution reagent were selected as potential α-amylase producing strains. Among the isolated strains**,** 21 α-amylase positive

strains were selected by exposing the agar plates to 1% iodine solution. Based on differences in enzyme production by clear zone on the plates, the highest α-amylase producer was selected for further investigation.

The morphological study, the microscopic observation and biochemical characterization revealed the presence of *B. subtilis* (STEP-5, MK 7, SMM-6), *B. macerans* (SMM-9), *B. megaterium* (MK-5 and TMP-2), *B. amylolequifaciens* (STEP-3 and SMM-4) and *B. licheniformis* (SW-6 and LAK-4**).** All the isolated *Bacillus* were gram positive, rod-shaped, aerobic, catalase positive and spore forming as described in by Barkeley [13] and Bergey's manual of determinative bacteriology (Table 1).

The obtained results from phenotypic identification indicated that selected α-amylase producing isolates was aerobic, Gram positive, rod shaped and spore forming. According to the biochemical results they belong to the genus *Bacillus.*

The α-amylase activity of *Bacillus* isolates according to their obtained hydrolysis halos is presented in (Table 1), which *B. subtilis* (A16) had the highest halo (3.1 mm), *B. macerans (*A6) and *B. coagulans* (A9) had the smallest (0.3 mm). Since the distinction between selected α-amylase producing strains lies in the soil and water origin, taxonomic characterization of isolates lead to explain that the amylolytic variation between the strains was not critical for species variability on the same microorganisms. Similar observations were made by [19] from α-amylase halos produced by different yeast strains isolated from starchy soil.

Table 1 : Illustration of α-amylase activity of the isolates strains belonging to the genus of *Bacillus*

Isolate code	Probable strains	Diameter of zone hydrolysis of α-amylase activity (mm)
STEP-3	*Bacillus amyloliquefaciens*	36
STEP-5	*Bacillus subtilis*	33
STEP-7	*Bacillus sp.*	35
STEP-9	*Bacillus sp.*	37
MK-5	*Bacillus megaterium*	31
MK-6	*Bacillus sp.*	36
MK-7	*Bacillus subtilis*	44
MK-8	*Bacillus sp.*	31
MW-5	*Bacillus sp.*	35
MW-6	*Bacillus licheniformis*	36
MW-9	*Bacillus sp.*	35
MW-10	*Bacillus sp.*	44
SMM-4	*Bacillus amyloliquefaciens*	33
SMM-6	*Bacillus subtilis*	44
SMM-8	*Bacillus sp.*	41
SMM-9	*Bacillus macerens*	39
LAK-4	*Bacillus licheniformis*	35
LAK-5	*Bacillus sp.*	37
LAK-8	*Bacillus sp.*	37
TMP-2	*Bacillus megaterium*	33
TMP-4	*Bacillus sp.*	38

Identification of the selected α-amylase producing MK-7 strain

The use of the phenotypic character for identification of the selected α-amylase producing MK-7 strain such as Gram positive, rod-shaped, positive catalase, spore formers and hydrolyzes of starch indicated that the isolates belong to the genus *Bacillus* (Figure 1, Figure 2). Furthermore, the selected α-amylase producing producer MK-7 strain was identified at the species level by the using of API 50CHB system as *B. subtilis*.

Optimization of the production medium and culture conditions

Optimizing the starch concentration

The obtained results of the influence of starch concentration on amylase production by the selected α-amylase producing MK-7 strain indicated that the maximum α-amylase yield 4.656 U/mL was obtained by the using concentration of starch of 0.5% (Figure 3). After that, amylase production was decreased considerably by further used increasing starch concentration and reached yield of 1.25 U/mL

Figure 1: Microscopic observation 100 × of the sele cted 24 hours old α-amylase producing strain MK-7, showing rod shape, Gram positive and spore forming. Bars = 10µm

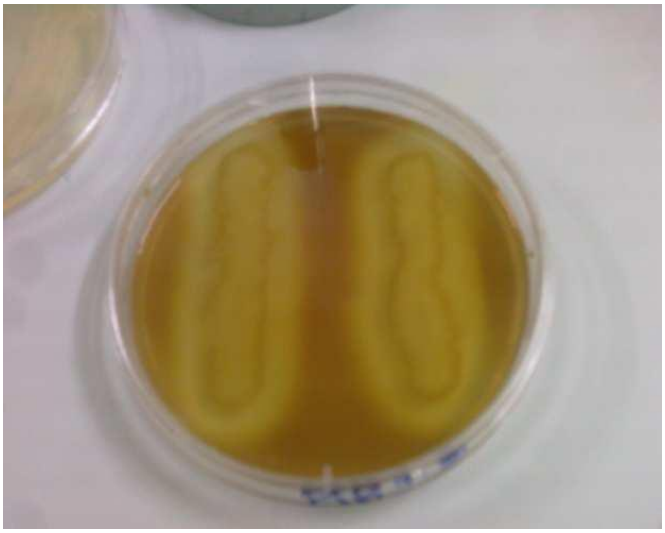

Figure 2: Streaks across the starch agar plate and the apparition of a clear zone around by the selected 72 hours old α-amylase producing MK-7 strain incubated at 37 °C for 72 hours

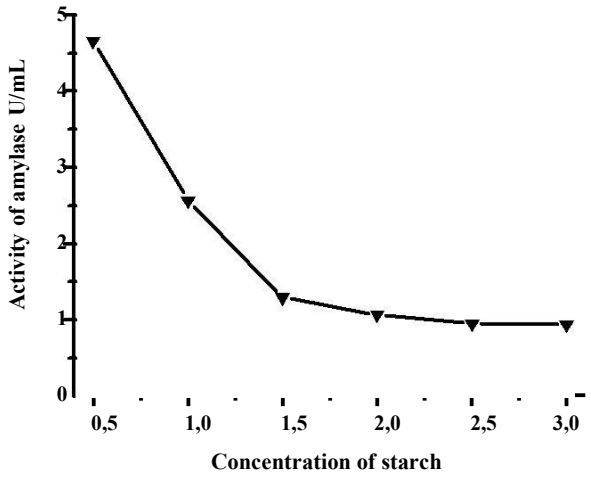

Figure 3: The optimization of the starch concentration for α-amylase production in the optimized culture medium by the selected α-amylase producing MK-7 strain incubated at 37 °C and agitated at 75 rpm for 72 hours

Optimization of the nitrogen source

A further study for the optimization of nitrogen source for α-amylase production of the selected α-amylase producing MK-7 strain has been investigated. The obtained results manifested that the highest α-amylase yield 7,036, 5.45 U/mL was obtained by the using of the gelatin and casein as nitrogen source respectively Whereas, the use of others nitrogen source such as urea and ammonium sulphate has decreased considerably the amount of amylase production (Figure 4).

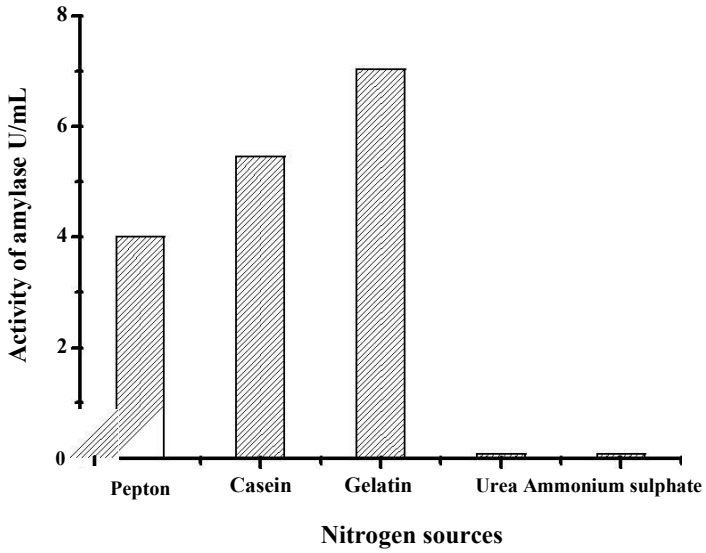

Figure 4:The optimization of the nitrogen source for α-amylase production in the optimized culture medium by the selected α-amylase producing MK-7 strain, incubated at 37 °C and agitated at 75 rpm for 72 hours

The effect of inoculum concentration

The obtained results of the influence of inoculation concentration on α-amylase production by the selected α-amylase producing MK-7 strain indicated that the maximum α-amylase yield 9,102 U/mL was obtained by the using concentration of inoculums of 0.5% (Figure 5). After that, amylase production has decreased considerably by the using of the increasing concentration inoculums and reached yield of 5.25 U/mL in the presence of 3 %.

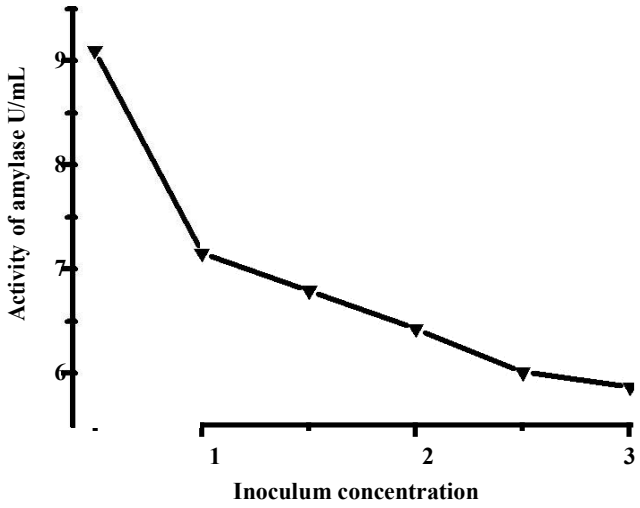

Figure 5: The effect of the inoculums concentration on α-amylase production in the optimized culture medium by the selected α-amylase producing MK-7 strain incubated at 37 °C and agitated at 75 rpm for 72 hours

The effect of metal ions on enzyme activity

The illustrated results in Figure 6 indicated that the inoculation of the selected α-amylase producing MK- 7 stain, in the presence of metals ions (Zn^{2+}, $Fe^{3+}Cr^{3+}$, $Fe3^{+}$) has reduced the enzyme accumulation in the optimized culture medium. Whereas, the presence of Mg^{2+} and Ca^{2+} in the optimized culture medium has increased considerably the yield of α-amylase activity 8,102 U/mL and 11,746 U/mL respectively.

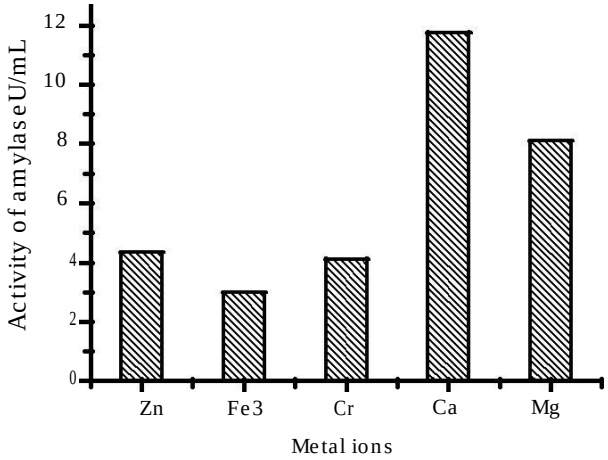

Figure 6: The effect of the metal ions on α-amylase production in the optimized culture medium by the selected α-amylase producing MK-7 strain incubated at 37 °C and agitated at 75 rpm for 72 hours

DISCUSSION

In order to isolate of α-amylase producer strains belonging to the genus *Bacillus*, the screening of the strains α-amylase producer strains *Bacillus* strain was carried out on starch agar medium, according the protocol described by [23, 25]**.**

Furthermore, the heat shock method was used as isolation strategy for the elimination of the vegetative cells and the enhancing the growth of spore-forming bacteria according to the protocol described by Lynn [17]**.**

Fifty four bacterial isolates belonging to *Bacillus* species were screened for α-amylase production on starch agar medium. Based on the criteria selection of α-amylase producer strains, two further selective steps such as a streak cross the Petri plates and DNS method were required.

The selected α-amylase producing MK-7 strain manifested a clear zone of hydrolysis of starch with a diameter of 49 mm and yield of 1.537 U/mL. The selected α-amylase producing MK-7 strain was identified as *B. subtilis* according to the reference of Bergey's Manual of Determinative Bacteriology [26] and by the using of the API system, therefore, this stain was retained for the optimization of further parameters implicated for α-amylase production. The α-amylase production has been thoroughly investigated, which was affected by several physicochemical factors. The most notable among these are the composition of the growth medium [2], Where the relationship between the starch concentrations present in the used optimized culture medium. The maximum α-amylase yields 4.656 U/mL was produced by *B. subtilis* MK-7 with a concentration of starch at 0.5% (w/v). Furthermore, a few α-amylase yields were obtained by the using of high concentration of starch. The obtained results is similar with the reported α-amylase production by *Bacillus* sp. IMD 370 described by Kalishwaralal and Kelly [27, 28, 29] by the using of starch concentration of 1%.

During the optimization of the nitrogen source for α-amylase production by *B. subtilis* MK-7, by the using of the organic nitrogen source as peptone, casein and gelatin and the mineral source such as ammonium sulphate and urea as been added in the optimized culture medium. The adding of the gelatin in the optimized culture medium has yielded the highest level of α-amylase production 7.036 U/mL, which has increased the productivity of α-amylase around 151%. Therefore, the gelatin was retained as appropriated source of nitrogen.

In early study, Gupta and their co-workers [2] has reported that the use of the organic nitrogen source such as yeast extract, peptone and casein was preferred of α-amylase production. Furthermore, the optimization of further parameters showed that the α-amylase production was mainly affected by inoculum concentration, which the maximum yield of α-amylase 9.102 U/mL was produced by *B. subtilis* MK-7 in the presence of inoculums concentration at 0.5% (v:v). After that, the α-amylase production was decreased in the presence of high inoculum concentration.

The study of the effects of different metal ions (Zn^{2+}, Cr^{3+}, Pb^{2+}, Fe^{3+}, Ca^{2+} and Mg^{2+}) on α-amylase production by the selected α-amylase producing MK-7 strain indicated that the adding of the calcium in the optimized culture medium has enhanced the yield of α-amylase 11.746 U/mL. A similar yield has been reported by Syed [10].

Furthermore, calcium ions are known to be the stabilizer and activator of α-amylase production and it has been reported that the Ca^{+2} were required for α-amylase activity.

Mamo and Gassesse [7] reported that a concentration of 0.1 g/L $CaCl_2$ was optimum for amylase production by *Bacillus sp. WN 11*, whereas Qader *et al.* [30] reported that in case of *Bacillus sp. AS-1*, 0.2 g/L $CaCl_2$ was optimum for the maximum production of α-amylase.

In the present study, the selected α-amylase producing MK-7 strain required calcium for enzyme production. This suggests that selected α-amylase producing MK-7 strain is calcium dependent metalloenzyme and possesses high affinity for calcium ions [2]**.**

Furthermore, the adding of magnesium in the inoculated optimized culture medium has manifested not a particular effect on α-amylase production. Whereas, the presence of the following metals ions: Fe^{2+}, Cr^{2+} and Zn^{2+} in the inoculated optimized culture medium have inhibited the α-amylase production. A similar result has been reported by Wanga and Asoodeh [31, 32].

CONCLUSION

Amylases are among the most important used enzymes in the industrial processes. With the advent of new frontiers in the biotechnology, the spectrum of α-amylase has a large application in the industrial application and therefore, it is of interest to search for new sources for the production of enzyme.

Newly, the isolated *B. subtilis* strain presented as excellent candidate producer for α-amylase in the optimized culture medium with the following composition: starch 5 g/L, gelatin 5 g/L $CaCl_2$, 0.2 g/L and 0.5% of inoculum size (v/v), with a maximum enzyme yield of 11.747 U/mL, which increase the yield of the α-amylase yields by 6 folds.

Furthermore, the obtained results indicated the existence of other interesting bacterial strains with important potential for α-amylase activity. Therefore, further studies characterization of the enzyme such as purification of the α-amylase and characterization of metal containing protein will require.

ACKNOWLEDGMENT

I would like to express my sincere thank to the Ministry of Higher Education and Scientific Research of Algeria for the financial support and Prof. Dr. Mouffok BENALI and Dr. BENINE Mohamed Lamine.

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