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OPTIMIZATION PARAMETERS FOR PRODUCTION OF A-AMYLASE BY *BACILLUS MEGATERIUM* KLMI4

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ABSTRACT
The present study reports on the optimization of process parameters for the production of α-amylase through submerged fermentation (SmF) by a bacterium, *Bacillus megaterium* KLMI4, in mineral medium amended with soluble starch. The organism was isolated from a cultivated arid soil of Kalaburagi district in the northern part of Karnataka State. The optimum levels of the parameters were observed to be: *p*H 8.0, temperature 37^0 C, inoculum size of $3x10^6$ cfu/ml per 100 ml mineral medium and substrate concentration of 1.5% soluble starch. The crude α-amylase was stable in the *p*H range of 7.0 to 9.5 and temperature range of 31^0 to 45^0 C. After optimizing the parameters, α-amylase production was observed to be enhanced by almost 6 times over that observed before optimization.

KEYWORDS: α-Amylase, starch hydrolysis, *B. megaterium* KLMI4, process optimization, submerged fermentation.

INTRODUCTION

Amylases are a group of extracellular enzymes like, α-, β- as well as glucoamylases that progressively hydrolyze the oligosaccharide molecules by random action or multiple attack action pattern into various end products like the dextrins and smaller polymers composed of glucose units. They are ubiquitous in nature being produced by plants, animals and microbes. They are amongst the major enzymes that find applications in a wide array of industries[1,2,3] and α-amylase is also reported to assist in reducing $AgCl_4$ into Ag-NPS or Ag-nanoparticles.[4] The first industrial production of amylase commenced in 1894 employing *Aspergillus oryzae*. For the industrial production of enzymes, especially amylases, microbial sources and mainly species of the genus *Bacillus* are preferred over others due to short fermentation cycle, protein secretion into the ambient medium, safe handling, eco-friendliness, easy manipulation to obtain required end product, adaptability to a wide array of ambient conditions, cost effectiveness and ease of process modifications as well as optimization.[5,6,7] Because of these amicable characters members of the genus *Bacillus* are generally deemed to be bacterial workhorses for the production of a variety of enzymes as well as fine biochemicals over the decades.[8,9,10]

Amylases have gained much importance in the present context and occupy nearly 25-30% of the world enzyme market.[11,12] The industrial enzyme market, valued at USD 4.2 billion in 2014, is estimated to reach USD 6.2 billion by 2020.[13] Different species of *Bacillus*, most commonly *B. subtilis*, *B. amyloliquefaciens* and *B. stearothermophilus*, have been reported to produce approximately 60% of commercial enzymes.[14] Moreover, different species of *Bacillus* generally have generally similar growth patterns as well as enzyme properties. However, their general properties like thermostability, *p*H profiles, stability etc. and conditions for optimization of fermentation may vary [15] and at the same time, these features of strains of the same species may vary based on the conditions existing in the different mini- or macro-ecosystems from which they are isolated.

Optimization of process parameters like initial *p*H, temperature, initial inoculum and substrate concentration is an essential step in the process of fermentation of a substrate, through either submerged (SmF) or solid state (SSF), to obtain the maximum possible yield of the desired enzyme or the end product.[3,10,16-22] Among the physical parameters, *p*H and temperature of the medium play an important role in α-amylase production and stability.[3] Cell growth and α-amylase production by *Bacillus* species and strains are considered to be dependent on the strain, fermentation methods, incubation period, cell growth, *p*H, temperature, inoculum size as well as substrate concentration in the fermenting medium.

The ever-growing demand for α-amylase has led to untiring and never-ending efforts to search for the bacterial species or strains capable of producing the enzyme. As a result of such an effort, a strain of *Bacillus megaterium* KLMI4 that was isolated from the cultivated arid soils of Kalaburagi District in Karnataka was observed to produce fairly good quantity of α-amylase.[23] The present study attempts to optimize process parameters of submerged fermentation (SmF) for producing α-amylase in enhanced quantities by *B. megaterium* KLMI4 and thereby to understand its potential for biotechnological application.

MATERIALS AND METHODS

Maintenance of Stock Culture and Inoculum Preparation

The bacterium, *B. megaterium* KLMI4, was isolated from soil samples of a sorghum field. The stock culture was maintained on nutrient agar at 4^0 C. Cells from the stock were streaked onto a nutrient agar supplemented with starch (1%) and incubated overnight at 37^0 C overnight. Stock culture was developed by transferring a loopful of cells from 18-24 h culture into the nutrient agar broth and incubated at 37^0 C. One ml of the stock culture (approximately $3x10^6$ cfu/ml) was used as the inoculum for the studies.

Production of α-Amylase and its Assay

Amylase production by the strain was studied through SmF in 250 ml Ehrlenmeyer flasks. Autoclave sterilized 100 ml fermentation medium in the flask (1% soluble starch, 0.4% NH_4HPO_4, 0.1% KCl, 0.05% and $MgSO_4.7H_2O$ in 100 ml distilled water and *p*H 7.2±0.1) was aseptically inoculated with 1 ml inoculum and incubated at 37^0 C in a rotary shaker incubator at 200 rpm. Samples were withdrawn from the flasks at intervals of 24 h to estimate the enzyme activity. Studies were conducted in triplicates. α-Amylase assay was done as per the DNS method[24] and the absorbancy was read at 540 nm in a double beam spectrophotometer (Systronics M2011). One unit (IU) of amylase was defined as amount of enzyme liberating one mole of reducing sugar per min.

Optimization of Process Parameters of SmF

Optimization of the important parameters governing the process of submerged fermentation, i.e., initial *p*H, temperature, inoculum size and substrate concentration, was done sequentially. Once a parameter was optimized, it was retained in the subsequent studies conducted to optimize other parameter. Initial *p*H of the fermentation medium was adjusted employing 0.1 N NaOH and 0.1 N HCl. Studies were conducted in the *p*H range of 5.0 to 10.0 with intervals of 0.5. Influence of temperature on α-amylase activity was evaluated in the range of 21^0 C to 49^0 C, with intervals of 2^0 C. Influence of initial inoculum was studied in the range of $1.0x10^6$ cfu/ml to $6.0\ x10^6$ cfu/ml. Influence of the substrate concentration was evaluated by varying the concentration of starch from 0.5% to 6.0% in the basal medium. All the studies were carried out in triplicate.

Enzyme stability at different *p*H

The crude enzyme fractions were maintained at different *p*H levels ranging from 5.0 to 10.0 employing citrate-phosphate buffer for *p*H 5.0 to 5.5, phosphate buffer for *p*H 6.0 to 7.5, tris-HCl buffer for *p*H 8.0 and glycine-NaOH buffer for *p*H 8.5 to 10.0 and incubated at 37^0 C for a period of 1 h and then enzyme was assayed for its activity as per the DNS method. The results are presented as relative activity.

Thermo-stability of the enzyme

The crude enzyme fractions were maintained at *p*H 8.0 employing tris-HCl buffer in different temperature conditions ranging from 21 to 49^0 C for a period of 1 h and then assayed for activity as per the DNS method. The results are presented as relative activity.

RESULTS AND DISCUSSION

The present study pursues the objective of optimizing the process parameters of submerged fermentation for production of α-amylase by *B. megaterium* KLMI4. Preliminary studies were carried out over a period of 120 h to estimate production of the enzyme by the bacterium in the mineral medium amended with 1% starch as substrate. Slow and gradual growth of the organism as well as α-amylase yield occurred reaching a maximum of 16 IU/ml at 48 h, thereafter decreasing sharply till 120 h (unpublished data). Hence further experiments were conducted over a period of 96 h. However, the enzyme activity expressed herein is the value obtained after 48 h of incubation.

In any microbial fermentation process from which a desired end product is expected, the basic step to be attempted for its maximal production is the optimization of the initial *p*H, temperature, initial inoculum and substrate concentration of the fermenting medium. As such, optimum *p*H for production of α-amylase was determined in the range of 5.0 to 10.0. Neither growth of the organism nor α-amylase production was observed at *p*H 5.0 and 5.5. Amylase production which was very less at 6.0, exhibited sharp increase as *p*H increased from 6.5 to 7.0 and reached maximum at 8.0 and thereafter it declined. The decline was very sharp after *p*H 9.0 (Fig. 1). The enzyme production appears to be stimulated at *p*H 7.0 and is suppressed as *p*H increases above 9.0, as observed by earlier workers.[25] Thus, *B. megaterium* KLMI4 appears to require slightly alkaline *p*H for α-amylase production.

Growth, morphology as well as metabolic activities of the microorganisms are determined to a great extent by *p*H since they are sensitive to the hydrogen ion concentration in the medium. It influences synthesis and secretion of the amylase and its stability as well.[26] Enzyme production by *B. megaterium* KLMI4 is very low in the acidic ranges and appears to be stimulated by alkalinity in the medium, recording optimum production at *p*H 8.0. However, *p*H levels above 9.0 decreased the amylase production substantially. As such, the

production of amylase by this strain appears to be favoured by slightly alkaline *p*H levels. The stability of the enzyme was good within the range of *p*H 7.0 to 9.5 (Fig. 1a). Our results agree with those of Shanmughapriya et al.[25]

Temperature greatly influences growth of the microorganisms. Though the present organism exhibits some growth at 21^0 C, amylase production at this temperature is very less. As temperature increases, amylase production increases; more so, after 29^0 C and it reaches maximum at 37^0 C (73 IU/ml). Its production decreased after 39^0 C. The enzyme activity was more than 50% in the range of 31^0 to 43^0 C, after which it sharply decreased as temperature increased to 49^0 C (Fig. 2). As such the present bacterium is a mesophile active in the range of 31 to 43^0 C. The crude enzyme has been observed to be stable in the range of 31 to 45^0 C, exhibiting more than 50% activity. Outside this range, the enzyme activity and stability are highly decreased (Fig. 2a).

Generally, different optimum *p*H levels in slightly alkaline range and optimum temperature have been reported for α-amylase production and activity by different mesophile species and strains of genus *Bacillus* and other genera: *p*H 7.0 and 37^0 C for *B. amyloliquefaciens, B. subtilis* and *Bacillus* sp.,[27] *p*H 8.0 and 35^0 C for *B. subtilis*,[28] *p*H 8.0 and 40^0 C for *Bacillus* sp,.[29] *B. subtilis*,[30] *B. licheniformis*,[31] and *Halobacterium salinarum*,[25] *p*H 8.0 and 37^0 C for *B. subtilis*,[10] as well as *p*H 6.5 to 40^0 C for *Geobacillus thermodenitrificans*.[32] It has been opined that mesophiles with mildly alkaline optima for amylase would find applications in detergent industries.[31] Thus amylase of *B. megaterium* KLMI14 may find applications in textile and detergent industries, apart from its other feasible uses.

The optimum initial inoculum size for maximal amylase production by *B. megaterium* KLMI4 has been observed to be $3x10^6$ cfu/ml. Enzyme production increased with the inoculum size, reaching maximum at inoculum of $3x10^6$ cfu/ml and declined in the higher ranges (Fig. 3). However the enzyme activity at the highest inoculum ($6x10^3$ cfu/ml) was very near to 50%. Inoculum size plays an important role in enzyme production in SmF.[33] Generally, the initial inoculum sizes vary from species to species as well as amongst the strains of the same species. As such, varying inoculum sizes (v/v) have been reported to be optimal for amylase production: 0.5%,[34] 2%,[10] 2.5%,[22] 3% ($7.5x10^8$ cfu/ml),[35] 4%,[36] 5%,[30] 10%,[37] and as high as 35%,[38] for various strains of *Bacillus subtilis* as well as 8.75% for *B. sonorensis*[39] and 5% for *B. cereus*.[30,34] Increasing the inoculum levels above the optimum proved detrimental to enzyme production as a result of rapid growth of the biomass and rapid consumption of essential nutrients as well as competition in the initial stages. Similar findings have been reported earlier.[40] The present results also suggest the same.

Influence of substrate concentration, i.e. of starch, on α-amylase production was studied by varying its concentration from 0.5% to 6.0% (w/v) in the medium. A concentration of 1.5% of starch has been noticed to be optimum for amylase production by *B. megaterium* KLMI14. As starch concentration increased from 0.5%, the enzyme yield reached 93 IU/ml at 1.5% and at higher starch levels a reduction in enzyme yield was noticed; but it was not as sharp as in other parameters, exhibiting an almost plateau like situation. At the highest (5%) concentration, nearly 75% enzyme production was still obtained (Fig. 4). Such a plateau-like situation has been reported earlier also at substrate concentrations above the optimum.[41-44]

Varying substrate concentrations have been observed to be optimum (w/v) for different strains of *Bacillus subtilis* have been reported: 0.5%,[45] 1%,[30] 2%,[46-48] and 4%.[49] The decline in enzyme activity at higher concentrations may be attributed to the high viscosity of the culture broth that would interfere with oxygen transfer leading to limitation of dissolved oxygen for growth of bacteria.[51]

With every step of parametric optimization of the SmF process (*p*H, temperature, inoculum size and substrate concentration), step-wise increase in α-amylase production at 48 h was observed (Fig. 5). Before optimizing any of the parameters, *B. megaterium* KLMI4 yielded 16 IU/ml at 48 h. In the first step of *p*H optimization, it rose to 60 IU/ml; further when temperature was optimized, the yield rose to 73 IU/ml. During optimization of the inoculum size, it was 76 IU/ml at $3x10^6$ cfu/ml; it is to be recollected that the same level of inoculum was being used in all the earlier studies and hence the difference between the second and third steps of optimization is negligible. When all the parameters were optimized (i.e., the fourth step), increased enzyme yield of 93 IU/ml was recorded, almost 6-times increase in α-amylase production over that obtained before optimization of any of the parameters.

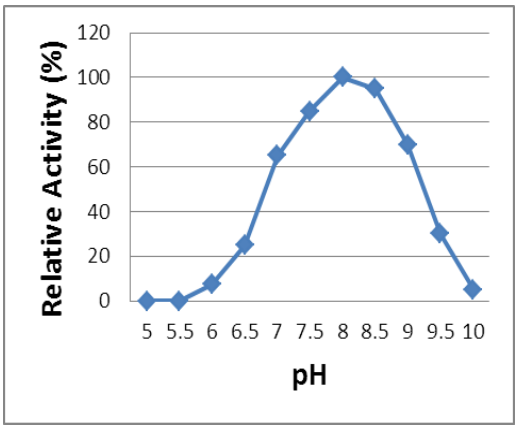

Fig. 1a. Influence of *p*H on α-amylase activity.

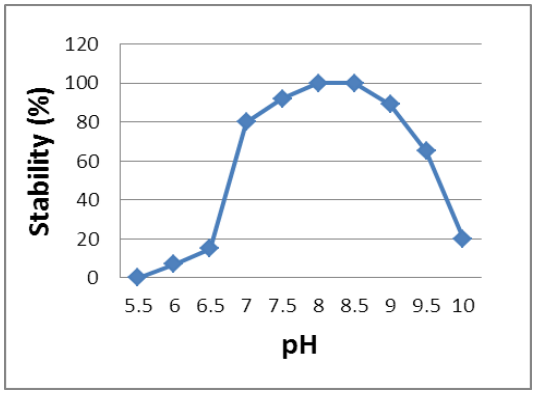

Fig 1b: Influence of *p*H on crude α-amylase stability.

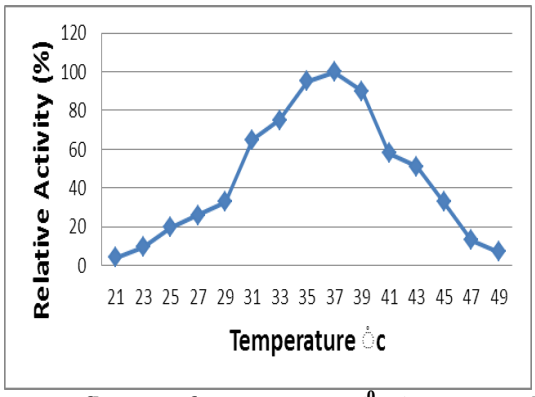

Fig. 2a: Influence of temperature (^{0} C) on α-amylase activity.

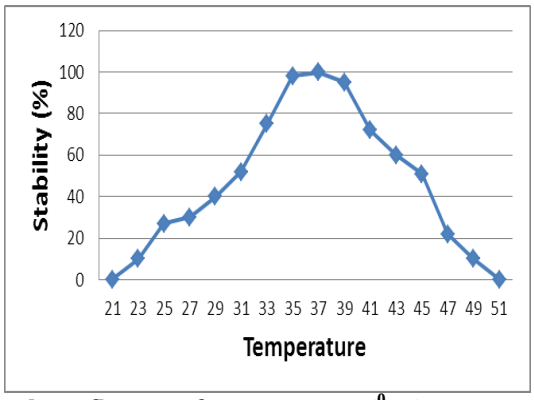

Fig. 2b: Influence of temperature (^{0} C) on crude α-amylase stability.

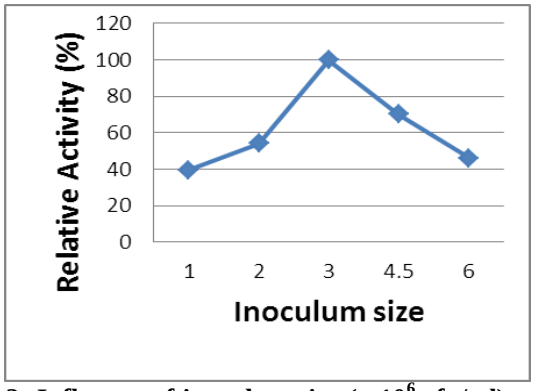

Fig 3: Influence of inoculum size (x 10^6 cfu/ml) on α-amylase activity.

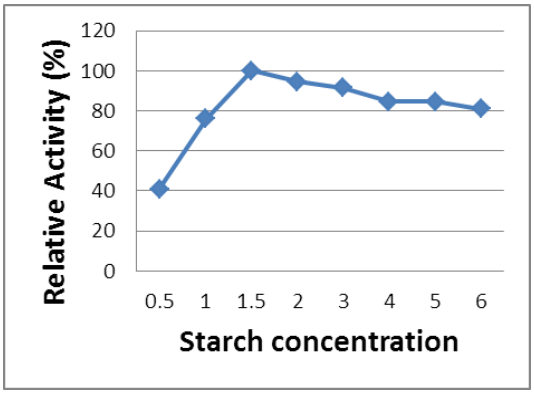

Fig 4: Influence of Starch concentration (%, w/v) on crude α-amylase activity.

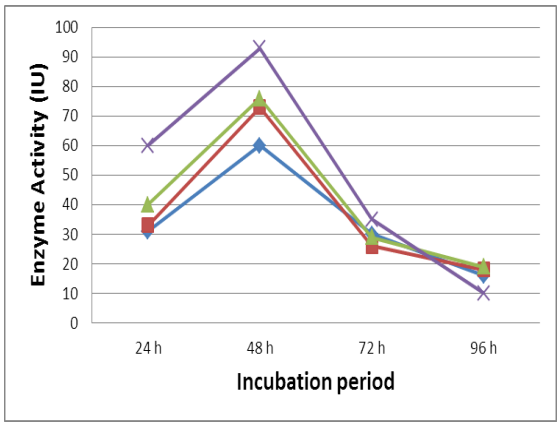

Fig. 5: Production of α-amylase with sequential optimization of process parameters. pH 8.0(◆); Temperature 37^0 C (■); Inoculum size 3x10^6 cfu/ml(▲); Starch 1.5% (×).

CONCLUSIONS

Once an organism capable of producing α-amylase is isolated, it becomes obvious to optimize the culture or fermentation conditions in order to obtain its maximum production. With this objective, the important process parameters of submerged fermentation of the starch in mineral medium for production of α-amylase by *Bacillus megaterium* KLMI4 were optimized in shake flask cultures in the laboratory. The optimum level of *p*H was found to be 8.0 and that of temperature was observed to be 37^0 C. The optimum inoculum size was noticed to be 3X10^6 cfu/ml and the optimum initial substrate concentration (sol. starch) was observed to be 1.5% (w/v) of the fermenting medium. Almost 6-fold increase in α-amylase production could be obtained after these parameters were optimized when compared to that before optimizing any of the process parameters. The bacterium, *Bacillus megaterium* KLMI4, has good potential of producing α-amylase and hence can be exploited for this purpose after further process economization through supplementing different C and N sources to the medium as well as employing cheaper substrates.

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