EFFECT OF VARIOUS PARAMETERS ON THE PRODUCTION AND ACTIVITY OF α–AMYLASE BY TWO *Bacillus* Spp. Namely *B.amyloliquefaciens* MTCC 1488 And *B.licheniformis* MTCC 1483 USING HOUSEHOLD ARGO-WASTES AS A SUBSTRATE IN SOLID STATE FERMENTATION.

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ABSTRACT

The objective of this work was to evaluate the "Effect of various process parameters on production and activity of α–Amylase by two *Bacillus* spp. namely *B.amyloliquefaciens* MTCC 1488 and *B.licheniformis* MTCC 1483 using household vegetable wastes as a substrate in solid state fermentation". Maximum enzyme titer obtained was 3467 U/g with the optimized production parameters i.e. after 72 hours of fermentation at 37^0C with the initial pH 4.0, initial moisture of 85% when the substrate inoculated with *B.amyloliquefaciens* of 2-10^9 CFU/ml; whereas 4419 U/g in case of *B.licheniformis* with the optimized production parameters, the only difference lies in temperature at 50^0C with the initial pH 7.5, initial moisture of 70%. Supplementation with 1% soluble starch showed maximum enzyme production in both cases, more so by *B.licheniformis* (5647 U/g). Supplementation of different nitrogen sources showed no significant effect on enzyme production by both strains. Among metal salts, enzyme production in both cases increased only when substrate supplemented with 0.1% $Cacl_2$. Optimum α–Amylase activity of *B.amyloliquefaciens* was observed at 60^0C, pH 7.0 and in presence of 0.15% $Cacl_2$; whereas in *B.licheniformis* only difference lies in temperature i.e. 70^0C. However, both the strains of *Bacillus* retailed more than 90% enzyme activity upto 100^0C compared to control 50^0C.

Key words: Alpha Amylase, *Bacillus amyloliquefaciens*, *B.licheniformis*, Solid state fermentation, Household vegetable wastes, Optimization.

INTRODUCTION

Amylases are among the most important industrial enzymes and are of great significance in present day biotechnology. Among two major classes of amylases, alpha – amylase (endo-1,4-D-glucan glucohydrolase, E.C.3.2.1.1) are extracellular enzymes that randomly cleave the 1, 4-D-glucosidic linkages between adjacent glucose units in the linear amylose chain and ranks first in terms of commercial exploitation[1,2].

Solid state fermentation (SSF) appears promising due to natural potential and advantages they offer. SSF resembles the natural habitat of microorganism and is, therefore, the preferred choice for microorganisms to grow and produce value added products[3,4].

The processing of vegetables leads to large amount of organic residues which are a kind of agricultural wastes. These wastes are one of the causes of environmental pollution. Many microorganisms, that include bacteria also and which are suitable for SSF applications, resembles a natural environment for their growth involving these low cost starchy substrates as a medium for the cost–effective SSF process by the activity of amylases[1,5,6].

Among bacteria, a number of *Bacillus* spp. is widely used for α–Amylase production to meet the industrial needs for various applications[1,2]. Hence, optimization of various parameters is one of the most important techniques used for the overproduction as well as activity of enzymes in large quantities to meet industrial demands[7,8,9].

The present work i.e., effect of various process parameters on production and activity of α–Amylase will be carried out with *Bacillus amyloliquefaciens* MTCC 1488 and *B.licheniformis* MTCC 1483 using household vegetable wastes as a substrate in SSF conditions.

MATERIAL AND METHODS

Microorganisms – alpha amylase producing *Bacillus amyloliquefaciens* MTCC 1488 and *B.licheniformis* MTCC 1483 which were obtained from MTCC, IMTECH, Chandigarh, India, were used as biological material. These were grown on nutrient agar (Hi-media) slants with a p^H of 7.0 at 37^0 C and 45^0 C respectively for 48h. The fully grown slants were stored at 4^0 C and were subcultured every two weeks.

Substrate – Household vegetable wastes were sundried for about two days and then oven dried at 60^0 C for 4h and made to powder (0.1mm) and finally used as substrate as for SSF throughout the study.

Preparation of inoculum – A volume of 50 ml of nutrient broth (initial p^H 7) taken in a 250 ml Erlenmeyer flask and autoclaved at 121^0 C for 15 min. and finally was inoculated with a loop full of cells from a 24h old slant of these microorganisms and kept at 37^0 C and 45^0 C respectively in a BOD shaker at 150 rpm. After 18h of incubation, 1ml of this broth culture was used as the inoculum of two test organisms. By serial dilution and plating, the number of variable colonies in the inoculum was found to be 2-10^9 CFU/ml.

Solid state fermentation – SSF was carried out by taking 5g of dry substrates in a 250 ml Erlenmeyer flask to which mineral salt solution (initial p^H 7) containing: KH_2PO_4 2, NH_4NO_3 10, NaCl 1, $MgSO_4.7H_2O$ 1 in g/l was added to adjust the required moisture level (75% as control) and sterilized at 121^0 C for 20 min. The flasks were inoculated using 1ml of culture broth of test organisms and incubated under static condition at 37^0 C and 45^0 C respectively in BOD incubator as control parameter and enzyme production was checked after every 24h for 5d.

Enzyme extraction - Enzyme was extracted by mixing 50ml of 0.1M Phosphate buffer (p^H 7) with fermented substrate on a rotary shaker at 250 rpm for 1h. The content was filtered through muslin cloth, filtrate was centrifuged at 8000xg at 4^0C for 10min and the supernatant was used for enzyme assay. Dry mass of the SSF substrate was determined by drying in hot air oven at 60^0 C for 16h.

Enzyme assay - A–Amylase activity was determined by incubating a mixture of 0.5 ml of crude enzyme and 0.5 ml of 1% soluble starch dissolved in 0.1M phosphate buffer (p^H 7) at 50^0 C for 15min[10]. The reaction was stopped by adding 1ml 3,5-dinitrosalicyclic acid and then boiled in the waterbath for 10min and finally cooled at room temperature. The final volume was made up to 12ml with distilled water and the reducing sugar released was measured at 540nm. A separate blank, containing phosphate buffer in place of crude enzyme, was set up for each sample to correct the non-enzymatic release of sugars. One unit (U) of the α–Amylase activity was defined as the amount of enzyme that releases 1 micromole of reducing sugar as glucose per minute, under assay conditions and expressed as U/g of dry substrate. All the experiments were performed in triplicates.

Influence of various parameters on enzyme production –

Incubation temperature – The effect of incubation temperature on production of thermostable α–Amylase by *Bacillus amyloliquefaciens* MTCC 1488 and *B.licheniformis* MTCC 1483 was studied by incubating the SSF medium at 35, 40, 45, 50, 55, 60, 65, 70 and 75^0 C for 72h.

Incubation period - The effect of incubation period was determined by incubating the SSF medium inoculating with two test organisms for different incubation periods (24, 48, 72, 96 and 120h) at 37^0 C and 50^0 C respectively.

Initial p^H – The effect of initial p^H of the medium on the yield of α–Amylase was studied by growing both the test organism for 72h at different initial p^H between 3.0 and 9.0 with the optimized incubation temperature of two test organisms. The p^H was adjusted with 1(N) Hcl or 1(N) NaOH before using it for moistening SSF substrate.

Moisture content – The influence of initial moisture level on enzyme production for 72h in both cases with above optimised parameters of the process by varying the ratio (w/v) of the substrate to mineral salt solution for adjusting the required moisture level i.e. 55, 60, 65, 70, 75, 80, 85 and 90%.

Inoculum size – The effect of different size of inoculum of both test organisms (0.5, 1, 2, 4, 6 and 8-10^9 CFU/ml) was investigated for the production of α–Amylase with the above optimised parameters of the process.

Carbon sources – The effect of different carbon sources i.e. soluble starch, glucose, maltose and lactose on α–Amylase production by both strains of *Bacillus* was studied each at 1% (w/v) initial concentrations. Other optimized parameters of the process were kept same.

Nitrogen sources – Different organic i.e. casine, peptone, tryptone and inorganic nitrogen sources i.e. $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl was studied for the production of α–Amylase by both strains of *Bacillus.* All these sources were studied at 1% (w/v) initial concentrations. Other optimized parameters (i-v) of the process were kept same.

Metal salts – The effect of various metal salts i.e. $FeSO_4$, $MgSO_4$, $CaCl_2$, $CuSO_4$ and $ZnSO_4$ on production of α–Amylase by both strains of *Bacillus* was studied each at 0.1% initial concentrations. Other optimized parameters (i-v) of the process were kept same.

Effect of various parameters on activity of enzyme – This was done by using extracted α–Amylase from control SSF i.e. before optimisation of different process parameters.

Effect of temperature: To determine temperature activity profile for an α–Amylase enzyme, assay was carried out at 40,50(control), 60, 70,80,90 and 100^0C

Effect of pH: For determination of suitable pH range for enzyme activity, pH of enzyme assay buffers (0.1 M) was varied as 5, 6(acetate buffer); 7 (control), 8 and 9 (phosphate buffer).

Effect of Ca^{+2} ions: For determination of suitable concentration of Ca^{+2} ions for enzyme activity, the concentrations was varied as 0.05,0.1,0.15 and 0.2 %.

RESULTS

The production of enzyme was optimum after 72h (3114 U/g) in case of *Bacillus amyloliquefaciens* MTCC 1488 at 37^0C with control process parameter. However, highest amylase activity (3956 u/g) was observed at 50^0C with after 72h in case of *B.licheniformis* MTCC 1483 which reflects the fact that amylase activity drastically increased after 45^0C.

The enzyme production shows the gradual increase through 24, 48, and maximum at 72h with optimised incubation temperature, more so by *B.licheniformis.* However, the enzyme yield showed a gradual decrease on further extension of fermentation period.

The enzyme production was maximum when initial medium p^H was 4.0 in case of *Bacillus amyloliquefaciens (*3291 U/g) but more so by *B.licheniformis (*4132 U/g) when initial medium p^H was 7.5. However, results show that enzyme production was minutely changed from p^H 4-7.0 and thereafter decreased in both cases.

Enzyme production was found to gradually increase with moisture content. For *Bacillus amyloliquefaciens*, the maximum enzyme production (3467 U/g) was observed when the substrate moisture was set at 85%. In the case of *B.licheniformis*, optimum moisture level was found to be 70% (4419 U/g) for amylase production.

Lower inoculum, below $2\text{-}10^9$ CFU, resulted in lower enzyme yield. The maximum enzyme production in both cases was recorded when 1ml of inoculum containing $2\text{-}10^9$ CFU was used. Thus 1ml was used as inoculum for further studies in both cases.

Addition of soluble starch gave the highest enzyme production more so by *B. licheniformis* (5647 U/g) than by *B. amyloliquefaciens (*4982 U/g) followed by maltose and lactose respectively. However, glucose was found to repress the enzyme yield in both cases.

Supplementation of additional nitrogen sources in the present study was found to repress the enzyme production in both cases. Only a marginal increase was noted with the addition of peptone, NH_4NO_3 and NH_4Cl.

Alpha–amylase production was only increased in the presence of $CaCl_2$, more so by *B. amyloliquefaciens* (3532 U/g from 3467 U/g) than *B.licheniformis* (4501 U/g from 4419 U/g). Other metal salts had a potent inhibitory effect on the production of amylase for both *Bacillus* strains.

In this study, α–Amylase produced by both *Bacillus* spp. showed considerable enzyme activity from lower to higher temperature, more so by *B.licheniformis* (4152 U/g) at 70^0C whereas highest enzyme activity (3182 U/g) of *B. amyloliquefaciens* observed at 60^0C. Interestingly, both the species of *Bacillus* retained more than 90% enzyme activity up to at 100^0C compared to the control 50^0C.

Maximum enzyme activity showed by both the species of *Bacillus* at pH 7 (control) and thereafter sharp decline in the enzyme activity in both cases were noticed. Presence of Ca^{+2} ions improve the enzyme activity in both cases, maximum at 0.15% concentration of $Cacl_2$.

DISCUSSION

The influence of temperature on amylase production is related to the growth of the organism. In the present study as the temperature increased after 37^0C and/or 50^0C, there was minute reduction in the enzyme production upto 70^0C in both cases that also found to be significant for support of their thermophilic nature. In contrary to this result, was reported by Dhanasekaran *et al.*[11] who found that optimum amylase production was at 45^0C for free and immobilized cells of *Bacillus* spp.; whereas Anto *et al.*[12] reported that 55^0C was found optimum for production of this enzyme by *B.cereus.* However, a wide range of temperature ($35\text{-}80^0$C) has been reported by others for optimum growth and α–Amylase production by *Bacillus* spp.[9,13]

The incubation period is governed by characteristics of the both strain and also based on growth rate and enzyme production. The decrease in enzyme yield after optimum level may be because of both the species have entered their late stationary phase i.e. both have a short lag and initial stationary phase and/or denaturation or decomposition of α–Amylase due to interaction with other components like secondary metabolites in the medium as it is also reported elsewhere[14,15].

Among the other parameters, the p^H of the growth medium plays an important role in enzyme secretion i.e. the production of α–Amylase is very sensitive to initial p^H of the fermentation medium[7,12]. Results of this study shows that enzyme production was maximum and generally stable from pH 4-7.0 which may indicate the buffering property of the substrate used for SSF[15,16].

The moisture content is an important factor that influences the growth and product yield in SSF. As the moisture content of the SSF medium changes during fermentation due to evaporation and metabolic activities, optimising the moisture level of medium is therefore most important. The optimal moisture level for maximum enzyme production *B. amyloliquefaciens* and *B.licheniformis* when the substrate moisture was set at 85% and 70 % respectively[8,12,16]. This necessary moisture content in SSF of both test organisms may exist in absorbed or complex form within the solid matrix, so that the entire system remained in solid state which is likely to be more advantageous for growth because of the increasing diffusion of solutes, absence of any free water and possible efficient oxygen transfer process.

There was a gradual decrease in the enzyme yield in both cases when size of the inoculum was increased from 2 to 8 – 10^9 CFU/ml. Varying inoculum size of bacterial cells during the fermentation indicated 2 - 10^9 CFU/ml inoculums as optimum for the enzyme production. Increase in inoculum size was found to adversely affect the enzyme production[8], may be due to the limiting nutrients.

Result indicates that α–Amylase is an inducible enzyme which is generally induced in the presence of carbon sources i.e. mainly in case of starch or its hydrolytic product maltose[15-17]. Easily metabolizable carbohydrates i.e. lactose may result in the better growth of bacteria along with reduction in the enzyme formation[9,18]. However, the reducing sugar glucose was found to repress the enzyme yield due to feedback inhibition and/or catabolic repression.

Supplementation of peptone, NH_4NO_3 and NH_4Cl were found to enhance the enzyme production marginally, but decreased when using other nitrogen sources in both cases[16,17]. However, others reported that various nitrogen sources, mainly organic sources induced growth and support maximum α–Amylase production by various *Bacillus* spp.[15,19]

As most α–Amylase s are known to be mettalo-enzymes, so supplementation of metal salts may provide good growth of *Bacillus* spp. and thereby better enzyme production. However, partial significant effect on enzyme production noticed only in the presence of $CaCl_2$, whereas others had a potent inhibitory effect in both cases[20].

As starch liquefaction is generally carried out at higher temperatures of 70-90^{0}C, the thermostable α–Amylase s are of great significance[13]. In the present study, α–Amylase produced by both strains showed a prominent enzyme activity as well might be stability also from 50-100 ^{0}C that comparable with other thermostable α–Amylase producing *Bacillus* strains[18,21].

The activity of the α–Amylase enzyme produced by both strains of *Bacillus* was maximum at p^H 7.0, but the use of alkaline buffer for enzyme reaction resulted in a sharp decline in the enzyme activity, may be due to the stability of the enzyme[22].

Ca^{+2} may have an effect on amylase activity and stabilization of the enzyme α–Amylase that protect the enzyme from denaturation by the activity of enzyme Proteases[1]. Thus, presence of Ca^{+2} ions may be improved the thermal stability as well as enzyme activity of both strains of *Bacillus*[12].

The results obtained in the present study with the effect of different process parameters on enzyme production as well as on enzyme activity indicated *Bacillus amyloliquefaciens* MTCC 1488 and *B.licheniformis* MTCC 1483 as a potential strains for production of α–Amylase using solid state fermentation with household vegetable wastes. Significant observation was that both strains showed and retained more than 90% enzyme activity up at 100^{0}C compared to the control at 50^{0}C. This makes the α–Amylase enzyme of both these two strains of *Bacillus* useful for various industrial applications particularly starch liquefaction at higher temperature.

Table 1: Effect of incubation temperature on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Incubation Temp /^{0}C	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
35	3041	3848
37	3114	-
40	3097	3862
45	3073	3879
50	3058	3956
55	3039	3943
60	3017	3939
65	3005	3924
70	2990	3912
75	2984	3895

Table 2: Effect of incubation period on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Incubation Period /h	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
24	944	1549
48	1869	2778
72	3114	3956
96	2003	2892
120	917	1463

Table 3: Effect of initial medium pH on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

pH	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
3.0	2629	3297
3.5	3012	3448
4.0	3291	3662
4.5	3194	3727
5.0	3163	3735
5.5	3156	3784
6.0	3127	3811
6.5	3119	3831
7.0	3114	3956
7.5	2913	4132
8.0	2654	3649
8.5	2322	3375
9.0	2149	2964

Table 4: Effect of initial moisture content of the SSF medium on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Initial moisture %	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
55	1806	2983
60	2432	3678
65	2605	3891
70	2892	4419
75	3114	3956
80	3343	3792
85	3467	3225
90	2501	2847

Table 5: Influence of inoculum size on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Inoculum size (CFU/ml)	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
$0.5\text{-}10^9$	2436	3518
$1\text{-}10^9$	2704	3704
$2\text{-}10^9$	3467	4419
$4\text{-}10^9$	3243	4191
$6\text{-}10^9$	3195	4074
$8\text{-}10^9$	3127	3892

Table 6: Effect of supplementation of carbon source on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Carbon source / %	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
Control (without carbon supplementation)	3467	4419
Soluble starch	4982	5647
Glucose	2912	3822
Maltose	3826	4794
Lactose	3454	4413

Table 7: Effect of supplementation of nitrogen source on α-amylase production by *B.amyloliquefaciens* and *B.licheniformis*

Nitrogen source / %	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
Control (without nitrogen supplementation)	3467	4419
$(NH_4)_2SO_4$	3375	4298
NH_4NO_3	3512	4486
NH_4Cl	3503	4469
Peptone	3529	4512
Casein	3416	4387
Tryptone	3434	4395

Table 8: Effect of temperature on activity of α-amylase produced by *B.amyloliquefaciens* and *B.licheniformis*

Temperature / ^0C	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
40	2967	3517
50	3114	3956
60	3182	4074
70	3153	4152
80	3091	3981
90	3006	3943
100	2985	3709

Table 9: Effect of pH on activity of α-amylase produced by *B.amyloliquefaciens* and *B.licheniformis*

pH	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
5.0	2743	3678
6.0	3096	3933
7.0	3114	3956
8.0	2945	3729
9.0	2802	3695

Table 10: Effect of Ca^{+2} on activity of α-amylase produced by *B.amyloliquefaciens* and *B.licheniformis*

Concentration of Ca^{+2} (%)	Enzyme activity / (U/g)	
	B.amyloliquefaciens	*B.licheniformis*
0.05	3187	3985
0.10	3230	4051
0.15	3324	4144
0.20	3105	3943

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