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Effect of differential production media on crude amylase produced by Novel *Bacillus Subtilis* sp. using submerged fermentation

Itoandon E E
Department of Research and Development, Almon Research Foundation Chennai, Tamil Nadu, India

Abstract
A preliminary investigation was carried out on the effect of different production media on amylase activity using submerged fermentation technique. A newly *Bacillus subtilis* sp. was constructed using *in vivo* modulation technique involving cloning of amylase gene insert with a designed 5'–3' forward primer (AAGGAGACGCGTATGTTTGCAAAACGATTCAAAACC) and 3' – 5' reverse primer (ATGTGATCTAGAATGGGGAAGAGAACCGCTTAAG) carrying an efficient targeted aprE subtilisin signal peptide bond. Luria Bertani (%), Peptone broth (%) and Pikovskaya broth (%) were used for amylase production by submerged fermentation technique. The results obtained showed that at optimal temperature 60ºC and pH 6.0 respectively; Pikovskaya broth gave the best amylase activity of 4.3 (U/ml) compared to amylase extract from Luria Bertani and Peptone broth respectively. *Bacillus subtilis* RIK 1285 was used as control to estimate the functionality of the newly constructed strain. The experiment showed how different factors such as nitrogen and carbon sources as well as trace elements can affect a microbial expression within a reaction.

Keywords: *Bacillus subtilis;* amylase; culture media; fermentation

Introduction

Alpha amylases are one of most important and widely used enzymes with wide range of application in many sectors beside their use in starch liquefaction and saccharification. They also find applications in food, baking, brewing, detergent, textile and paper industries. Increasing utility and consumption of amylase in different industries has placed a greater stress on increasing indigenous enzyme production and search of more rapid processes (Carlsen *et al.*, 1996; Kathiresan and Manivanan, 2006) [5]. Amylase is among the most important enzyme used in biotechnology and originate from different sources (plants, animals and microorganisms), (Burhan *et al*., 2003) [4]. The synthesis of these enzyme by microorganisms has been reported to be highly influenced by factors such as carbon sources, temperature, pH, and operating parameter such as incubation time in submerged culture giving higher yield as compared to other techniques (Jacob and Prema, 2006) [9]. Factors like carbon, nitrogen sources and their concentrations have always been of great interest to researchers in the industry for the low-cost media design. It is also known that 30–40% of the production cost of industrial enzymes is estimated to be the cost of growth medium. Therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production. High fermentation medium cost is one of the major concerns in amylase production from microbial sources. Amylase demand is continuously increasing and researchers are attempting to establish economical fermentation processes. Several agro-industrial residues have been utilized for amylase production and many other value-added products. One extremely valuable advantage of conducting biotechnological processes at elevated temperatures and low pH is controlling the risk of contamination by common mesophiles. Allowing a higher operational temperature and low pH also has an appreciable influence on the solubility and bioavailability of organic compounds and thereby provides efficient bioremediation. Other advantages of carrying out industrial processes at elevated temperatures and low pH include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and favourable equilibrium displacement in endothermic reactions (Aguilar *et al*., 2000) [1]. Such enzymes could also be used as models for the understanding of thermostability and thermoactivity, which is also useful for protein engineering (Annamalai *et al*., 2011) [2]. Various reports have appeared on the microbial synthesis of amylase on different media (Kundu and Das, 1970) [11]. Optimization of medium by the classical method involves changing one independent variable keeping the other factors constant. In fermentation process, the operational variables interact and influence each other. As a result, it is important that the optimization method accounts for the interactions so that a set of optimal experimental condition can be determined (Silva and Roberto, 2001). It is necessary to understand the effect of media composition on microbial activities during synthesis of secretomes. The aim of this investigation is to validate and express the significant effect of media composition on optimal activity of newly constructed strain of *Bacillus subtilis* sp.

Materials and Methods

1. Construct of *Bacillus subtilis* sp host

A complete genome of *Bacillus subtilis* 16s sequence was obtained from the National Center for Biotechnology Information (NCBI) database. The specific Forward and Reversed primers were designed having aprE subtilisin signal peptide targeted amylase gene insert using the SnapGene tool. The construct was done following the InFusion cloning manual (Chen *et al*., 2014) [7]. The following properties were considered: 1). 5' end of the primer contained 15 bases that are homologous to 15 bases

at one end of the insert. 2). 3' end of the primer also contained sequence that is specific to the target gene. 3). Other properties considered were GC-content between 40–60%, melting temperature (Tm) between 58–65°C with ≤ 4°C range.

2. Construction of Expression Plasmid

The following steps were carried out: Step 1). Construction of expression plasmid by inserting a target gene into the multicloning site downstream from the secretory signal peptide of pBE-2 DNA. Step 2). Using the restriction enzymes MluI and Eco521 to completely digest the expression plasmid constructed in Step 1. Step 3). Preparation of In-Fusion reaction solution by mixing the DNA mixture and the expression plasmid already digested. Step 4). Mix the reaction solution by pipetting gently. Step 5). Allow the mixture to incubate at 50℃ for 15 minutes and then incubate on ice. Step 6). Thaw the Stella competent cells on ice immediately prior to their use. Step 7). After thawing the cells, mix gently to homogenize and transfer 100 µl of competent cells to a 14 ml rounded-bottom test tube. Do not vortex. Step 8). Add 2 µl of the In-Fusion reaction solution diluent from Step 5 to the tube in Step 7. Step 9). Incubate on ice for 30 minutes. Step 10). Incubate at 42℃ for 45 seconds. Step 11). Repeat incubation on ice for 1 to 2 minutes. Step 12). Add SOC culture medium pre-warmed to 37℃ to obtain a final volume of 1 ml. Step 13). Shake at 160 to 225 rpm for 1 hour at 37℃. Step 14). Spread a suitable volume on Luria Bertani (LB) agar plate containing ampicillin (100 µg/ml). Step 15). Incubate overnight at 37℃. Step 16). After suspending the colonies from the plate in LB and harvesting them, purify the plasmids to produce the plasmid library. A method of Nguyen *et al.*, 2005 was carried to obtain the above construct.

3. Primary screening for amylase production

The screening of newly constructed *B. subtilis* sp. trans-formants was carried out using plate assay having an inclusion of kanamycin (50µg/ml) and Luria Bertani agar (%). A commercial *Bacillus subtilis* RIK1285 was used to verify successful transformation and also comparable level of amylase synthesis on Luria Bertani agar witht inclusion of kanamycin (50µg/ml). Amylolytic degrading ability was carried out following the method of (Thippeswam *et al*., 2006) [18] and (Bahadure *et al*., 2010) [3].

4. Submerged production of amylase

The following differential media were prepared under sterile condition and used for production of amylase. The ingredients for each minimal medium are the following (%): (1). Luria Bertani broth (Tryptone 1.0g, Yeast extract 0.5g, NaCl 1.0g, Starch 1.0g). (2). Pikovskaya broth (Glucose 1.0g, $Ca(PO_4)_2$ 0.5g, $(NH4)_2SO_4$ 0.05g, $MgSO_4.7H_2O$ 0.001g, $MnSO_4.7H_2O$ 0.001g, $FeSO_4$ 0.001g, KCl 0.02g, Yeast extract 0.05g). (3). Peptone broth (bacteriological peptone 0.6g, $MgSO_4.7H_2O$ 0.05g, KCl 0.5g, soluble starch 1.0g). Submerged protocol for amylase production was carried out using 1.0ml of each microbial suspension (i). Newly constructed *Bacillus subtilis* sp. with aprE subtilisin signal peptide having amplified binding target amylase gene and (ii). *Bacillus subtilis* RIK 1285. A modification of (Kaur and Viyas, 2012) and (Sumrin *et al*., (2011) [17] was used for this purpose.

5. Determination of Temperature (ºC) and pH for amylase enzyme

A modification of Carrasco *et al*., (2016) [6] as described was followed: the reaction mixture prepared were incubated at various temperature of 30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC. To 1ml of the incubated samples with 0.5ml of the enzyme from the different temperature, 1ml of iodine was added and the resulting colour (blue black) was determined for its absorbance at 540nm. Different reaction mixtures prepared were adjusted to different pH values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 using 6N HCl and 5N NaOH solutions intermittently. One ml of each sample was mixed with 1ml of iodine added to it and incubated for 40 minutes at 30ºC. The resulting colour (blue black) was determined for its absorbance at 540nm. The concentration of reducing sugar release was then calculated for glucose.

Results and Discussion

Amylase stability is of a major interest to the various industries and as such production of thermos-stable amylase has become a common expectation. Since microbial amylase is still considered to be cheap and reliable, hence manipulation the target strain using microbial engineering techniques for optimal activity is also a major concern. The results obtained in this preliminary investigation are extracts from one of our current work but variable expression suggest the principle of microbial interaction with its immediate environment. The importance of amylase to the industries and even its simple home application cannot be overemphasize. Hence stability and yield are variable concerns. The Figures and Tables discussed below has effectively shown the level of interaction between strain and medium composition.

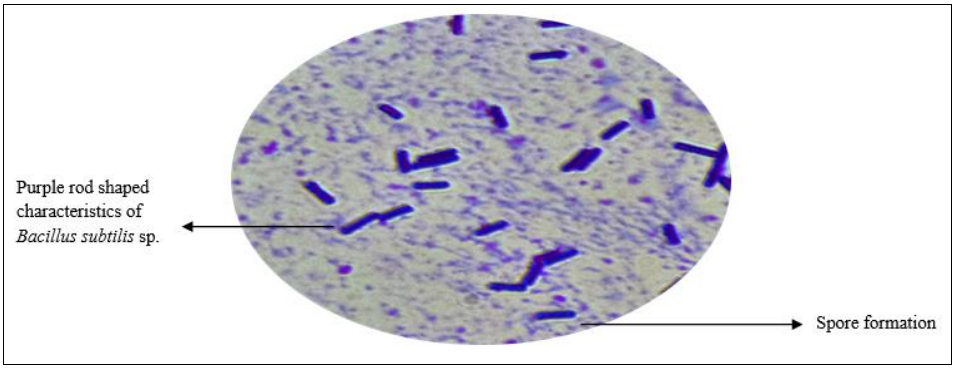

Fig 1: first picture

Morphological characteristics of newly constructed *Bacillus subtilis* sp with aprE subtilisin signal peptide bond grown on Luria Bertani agar fortified with 50µg/ml of kanamycin. The image was prepared by gram's reaction technique and observed using a fluorescence microscope of X100 magnification. The Figure above clearly shows the positive purple coloured rod shaped cells indication the presence of peptidoglycan with techoic acid allowing pigment retention. Spore formation which is another characteristic was observed by the new *Bacillus subtilis* sp as shown in the image above.

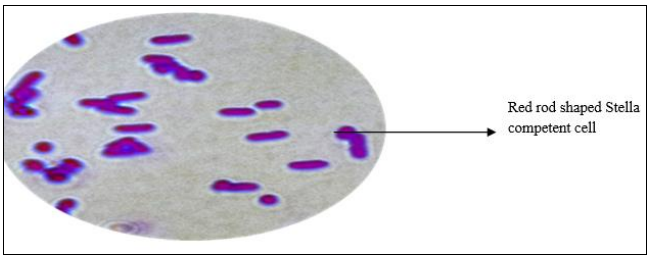

Fig 2: Second picture

Morphological characteristics of Stella competence cells grown on Luria Bertani agar fortified with 50µg/ml of ampicillin. The image was prepared by gram's reaction technique and observed using the fluorescence microscope of X100 magnification. Gram negative bacteria will stain red because the thin peptidoglycan layer is surrounded by the plasma membrane and thus will not retain pigmentation.

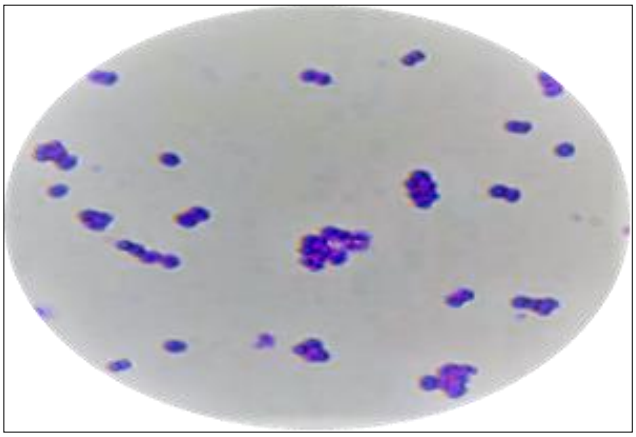

Fig 3: third picture

Cellular morphology of transformed *Bacillus subtilis* sp. The effect of kanamycin antibiotic on newly constructed *Bacillus subtilis* sp strain is shown in this image. From the figure, defensive meachanistic property of the strain was being expressed by forming a protective cyst. It is observed that there interaction between the strain and the antibiotic but the reaction was limited to shock expression by the strain.

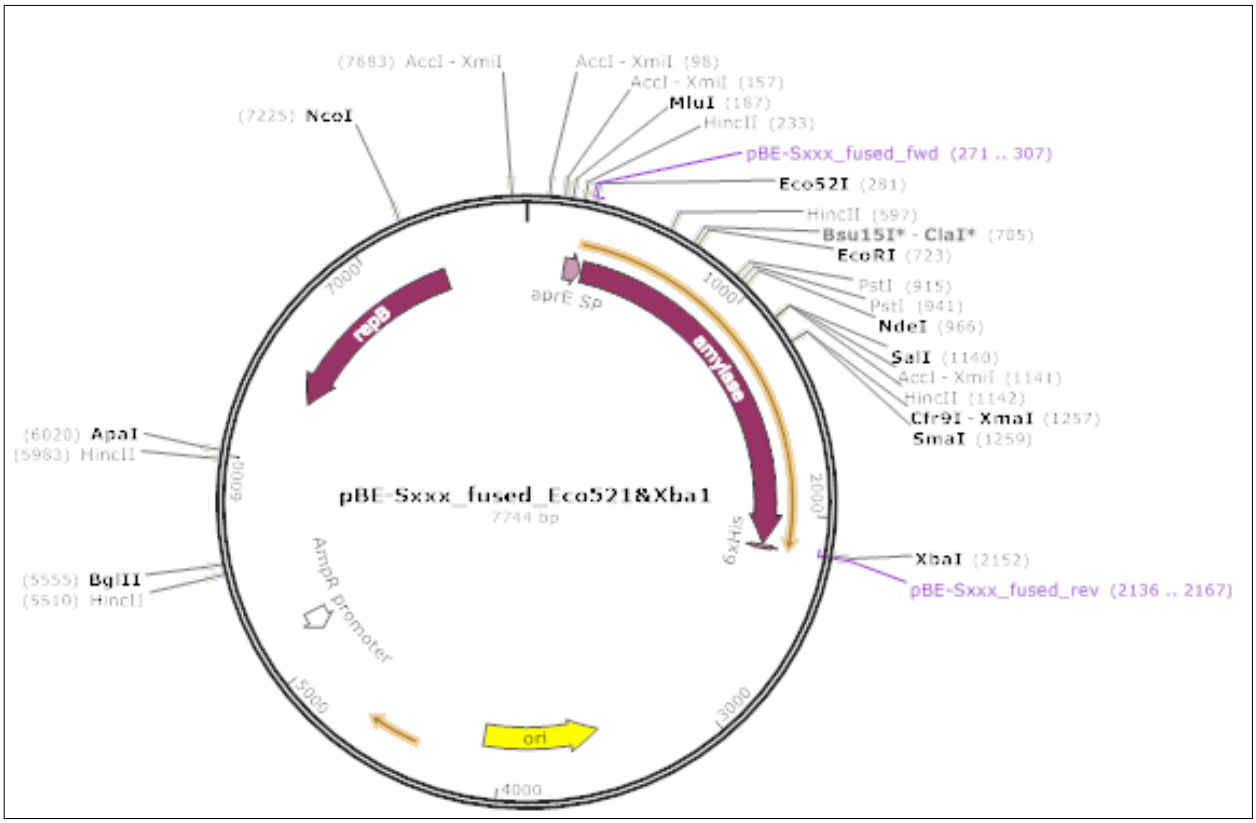

Fig 4: fourth picture

Geneotypic map of the newly constructed *Bacillus subtilis* sp.The Figure shows the successfully uptake of cloned formulation consisting of amplified targetted amylase gene + pBE-S DNA + signal peptide bond. The map is showing is showing increasing in size of over 7kbp as compared to the original size of over 5kbp of vector provided by the Takara In Fusion Cloning.

Fig 5: fifth picture

Positive screening for amylolytic property of newly constructed *Bacillus subtilis* sp. shown in 5a while 5b is showing negative screening result by *Bacillus subtilis* RIK 1285. The starch agar media used for screening had 50µg/ml kanamycin and as such only the successful strain was able to grow and furthermore degrade starch.

Production of amylase was carried by the newly constructed Bacillus subtilis sp. And Bacillus subtilis RIK 1285 using submerged fermentation. Three (3) different media compositions were used and subjected to same production condition after 24hours. The production conditions were 52ºC, pH 7.0 using a shaker incubator at 120 rpm. At the end of fermentation period samples of amylase were taken and assayed using the 3,5-dinitrosacyclic acid method to ascertain activity of enzyme at optimal temperature (ºC) and pH. The results obtained are shown and discussed below. From the Table 1 above it is pobserved that sample amylase produced by *Bacillus subtilis* sp. using Luria Bertani medium had an activity of 0.41 U/ml at 60ºC as compared to *Bacillus subtilis* RIK 1285 with activity of 0.33 U/ml at 50ºC. Similar reactions were obtained from samples extracted from Pikovskaya and Peptone media as shown in Table 2 and Table 3 respectively having 60ºC as optimal temperature. Amongst the three media Pikvoskaya medium composition gave the highest activity value of 0.43 U/ml. Assay for optimal pH was carried. Table 4, 5 and 6 shown below represent the activities at optimal pH obtained from amylase extracted from the different media as specified. From Table 4, samples of amylase produced by *Bacillus subtilis* sp from Luria Bertani was observed to have 0.40 U/ml activity at optimal pH 6.0.This was higher the sample from Bacillus subtilis RIK 1285 having 0.38 U/ml activity at optimal pH 7.0. Similarly, samples from Pikovskaya and Peptone media also had their highest activities at pH 6.0 for newly constructed *Bacillus subtilis* sp as shown in Table 5 and 6 respectively. It was also observed that Pikovskaya medium composition gave the best activity at lower pH.

Table 1: Effect of optimal temperature (ºC) on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Luria Bertani medium.

Temperature (°C)	LB *Bacillus subtilis* + aprE SP + pBE-S + amylase gene	LB *Bacillus subtilis* RIK 1285
30	0.28	0.25
40	0.34	0.29
50	0.38	0.33
60	0.41	0.26
70	0.38	0.24
80	0.32	0.19
90	0.27	0.17
100	0.23	0.15

Table 2: Data compilation for effect of optimal temperature (ºC) on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Pikovskaya medium.

Temperature (°C)	PK *Bacillus subtilis* + aprE SP + pBE-S + amylase gene	PK *Bacillus subtilis* RIK 1285
30	0.22	0.28
40	0.27	0.33
50	0.39	0.38
60	0.43	0.35
70	0.32	0.27
80	0.22	0.16
90	0.16	0.14
100	0.14	0.12

Table 3: Data compilation for effect of optimal temperature (ºC) on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Peptone medium.

Temperature (°C)	PM *Bacillus subtilis* + aprE SP + pBE-S + amylase gene	PM *Bacillus subtilis* RIK 1285
30	0.28	0.25
40	0.33	0.3
50	0.39	0.34
60	0.42	0.31
70	0.33	0.23
80	0.24	0.17
90	0.16	0.12
100	0.13	0.09

Table 4: Data compilation for effect of optimal pH on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Luria Bertani medium.

pH	LB *Bacillus subtilis* + aprE SP + pBE-S + amylase gene	LB *Bacillus subtilis* RIK 1285
3	0.22	0.19
4	0.3	0.25
5	0.35	0.3
6	0.4	0.35
7	0.37	0.38
8	0.3	0.32
9	0.23	0.21
10	0.16	0.14
11	0.13	0.11

Table 5: Data compilation for effect of optimal temperature pH on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Pikovskaya medium.

pH	PK Bacillus subtilis + aprE SP + pBE-S + amylase gene	PK Bacillus subtilis RIK 1285
3	0.23	0.19
4	0.3	0.23
5	0.38	0.29
6	0.43	0.33
7	0.36	0.35
8	0.3	0.28
9	0.24	0.22
10	0.18	0.17
11	0.15	0.14

Table 6: Data compilation for effect of optimal temperature pH on amylase enzyme produced by constructed *Bacillus subtilis* spp. and control using Peptone medium.

pH	PM *Bacillus subtilis* + aprE SP + pBE-S + amylase gene	PM *Bacillus subtilis* RIK 1285
3	0.18	0.16
4	0.25	0.23
5	0.32	0.29
6	0.39	0.33
7	0.36	0.35
8	0.31	0.26
9	0.26	0.2
10	0.21	0.15
11	0.16	0.13

Conclusion

Microorganism reaction to nitrogen sources, carbon sources, trace elements, promoter and substrates in a compounding medium as growth factors are very important to microbial growth and functionality be it wild or modified strain. Sequence to the amount of substantial quantity of these factors is still much being investigated. The investigation showed growth factors present in media with different effect on activities of a constructed *Bacillus subtilis* sp. Further work is required to understand the expression level of growth factors utilized by microorganisms for growth, development and optimal activity.

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