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Development of a solid-state fermentation process for production of bacterial α-Amylase from agro-byproducts and its optimization

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ABSTRACT

Solid-state fermentation (SSF) was carried out using different agro-byproducts as substrate for the production of α-amylase using a bacterial culture of Bacillus licheniformis. Among all the substrates wheat bran supported maximum growth and produced maximum alpha amylase (154.17 IU) under Triton –X as extraction medium. Production optimization was conducted using wheat bran. The maximum alpha amylase production was obtained at pH 6 (170.34 IU) 40°C (168.78 IU), 48 h (155.06 IU) and with 80% initial moisture (171.89 IU). Supplementation with 1% maltose further enhanced enzyme yield (178.46 IU). However, glucose and lactose inhibited the enzyme production. Increase in the enzyme yield (171.68 IU and 172.36 IU) was obtained when fermentation medium was supplemented with 1% soy peptone and (0.15 M) ammonium chloride, respectively. Addition of 0.01 M phosphate concentration also enhanced amylase production. Therefore, from the present study it can be concluded that starch digesting α- amylase produced by Bacillus licheniformis in solid state fermentation could be important in biotechnological application.

Keywords: SSF, Agro-byproducts, Enzyme activity, Wheat bran, Bacillus licheniformis

Alpha amylases (endo-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo enzymes that randomly cleave the 1,4-α linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units. α-amylases are used for the enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving

the production of glucose and maltose via further hydrolysis (Gupta et al., 2003; Prakash and Jaiswal 2009).

These enzymes are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups. Amylase has been employed in textile industries for long time for desizing processes that involves the removal of starch from the fabric to prevent breaking of the warp thread during the weaving process and in the pulp and paper industry for the modification of starch of coated paper to make the surface of paper sufficiently smooth and strong that improves the writing quality of the paper (Van der Maarel et al., 2002; Gupta et al., 2003; Couto and Sanroman 2006).

α- Amylase has been derived from several fungi, yeasts and bacteria however enzymes from fungal and bacterial sources have dominated applications in industrial sectors. (Pandey et al., 2000; Konsoula et al., 2007). Thermostability of an enzyme is a desired characteristic of most of the industries. Bacillus subtilis, Bacillus stearothermophilus, Bacilluslicheniformis, and Bacillus amyloliquefaciens are known to be good producers of thermostable α-amylase that have been currently investigated to improve industrial processes of starch degradation. (Gomes et al., 2003; Stamford et al., 2001; Asgher et al., 2007). Several reports of production have shown alpha amylase production from Bacillus sp. particularly Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis (Ashraf et al., 2005; Rameshkumar and Sivasudha 2011; Garg and Kaur 2013).

Mostly synthetic media have been used for the production of bacterial amylase through Smf. To meet the demand of industries, low-cost medium is required for the production of α-amylase. For the reduction of the cost of the medium the synthetic media could be replaced with cheaper agricultural by-products (Haq et al., 1997; Haddaoui et al., 1999; Hamilton et al., 1999). The agro-substrates such as sunflower meal, rice husk, cottonseed meal, soybean meal, and pearl millet and rice bran have been tried for SSF that can be defined as the growth of microorganisms on moist solid substrate with negligible free water (Nandakumar et al., 1996; Haq et al., 2003; Pandey et al., 2001; Baysal et al., 2003; Kunamneni et al., 2005; Verma et al., 2008). There are other advantages of SSF over SmF, including superior productivity, simpler technique; lower capital and recurring investment, lower energy requirement, lower level of catabolite repression, end product inhibition, less water output, better product recovery and lack of foam build up (Tanyildizi et al., 2005; Couto et al., 2006; Vishalakshi et al., 2009). Enzymes production was carried out by solid state fermentation and it had been established as a superior technique for the production of enzymes (El-Shishtawy et al., 2014)

Different physical and chemical factors such as temperature, pH, period of incubation, moisture and agitation, carbon sources acting as inducers, surfactants,

nitrogen sources, phosphate, different metal ions, have been known to affect the production of α- amylase with regards to SSF Therefore, optimization of processing parameters are one of the most important techniques used for the production of enzymes in large quantities. Several authors have described the modification of nutrients and cultured conditions for amylase production with various Bacillus strains using flask culture and batch fermentation (Hassan and Karim 2012; Serin et al., 2012; Singh et al., 2014; Rai and Solanki 2014)

There are several bioprocesses that have been developed utilizing the agro materials for the production of bulk chemicals and value-added fine products including enzymes. Solid-state fermentation (SSF) might be attractive technology for value-addition and utilization of cheaper agricultural residues for the production of enzymes. Thus the present study describes the evaluation of various agro-byproducts for α -amylase production under solid state fermentation and the optimization of α-amylase production under different processing conditions as well as medium components.

Materials and Methods

Place of Work

The present study was conducted in the Department of Microbiology and Fermentation Technology, Jacob School of Biotechnology and Bioengineering, Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed to be University), Allahabad (U.P).

Procurement and Maintenance of Culture

Bacillus licheniformis (MTCC 429) used in the present study was obtained from the Microbial Technology Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh (Punjab), India. The strain was grown on nutrient agar slants and maintained at 4°C.

Substrates and its pretreatment

Various agro by-products and their residues viz. Wheat flour, Barley flour, Corn flour, Gram flour, Moong husk, Arhar husk, Mustard oil cake, coconut oil cake, Banana peel, Potato peel, Sweet Potato peel, Soybean hull, Wheat bran, Rice bran, and Sugarcane baggase were used as substrate for solid state fermentation (SSF) and procured from local market of Allahabad city, India. Pretreatment of agro-residues were performed by the following method described by Asgar et al., (2002) by chopping agro residues like bran, peel and baggase and dried (70°C)

for 16 h. The dried residues were then ground to powder form (40 mm mesh) and stored in polythene bags at room temperature (30±2°C) till used as substrate for alpha amylase production.

Preparation of Inoculum

Bacterial Inoculum was prepared by the method described by Gangadharan et al., (2006). A volume of 50 ml of nutrient broth was inoculated with a loopful of cells from a 24h old slant and kept at 37°C in a rotary shaker (100 rpm). After 18 h of incubation, 1 ml of this nutrient broth culture of Bacillus licheniformis was used as the inoculum for solid state fermentation.

Solid state fermentation medium for production of Alpha amylase

Fermentation medium was prepared by the method described by Gangadharan et al., (2006). Five grams of each of the dried substrate were placed in 250 ml Erlenmeyer flasks and then moistened with mineral salt solution (K2HPO4, 2g/L; NH4NO3, 10g/L; NaCl, 1g/L; MgCl2, 1g/L). Distilled water was added to the mineral salt solution in order to maintain the moisture content of medium. The fermentation media in the flasks were autoclaved at 121°C for 20 minutes and cooled to about 30°C. The flasks were inoculated with 2% inoculum of Bacillus licheniformis and the contents of the flask were mixed thoroughly to ensure uniform distribution of the inoculum. The flasks were incubated at 37°C for 24 to 48 h in a shaking incubator operated at speed of 100 rpm. All the experiments were run parallel in triplicates.

Extraction of alpha amylase and its estimation

After fermentation, the fermented matter in each flask was extracted by the addition of different extraction medium like distilled water, 0.2M Phosphate buffer (pH 7±0.2), 0.1% Tween-80 and Triton-X-100 to a total extract volume of 100 ml. The entire content was mixed thoroughly at 30°C for 1 h in rotary shaker at 100 rpm and filtered using a Whatman filter paper no.1. The suspensions were then centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant was carefully collected and used as crude enzyme extract for the estimation of alpha amylase activity.

Estimation of alpha amylase

The alpha amylase assay was carried out according to the method described by Okolo et al., (1995). The reaction mixture consisted of 1.25 ml of 1 % soluble starch, 0.5 ml of 0.2 M phosphate buffer (pH=7), and 0.25 ml of crude enzyme

extract. After 10 min of incubation at 37°C, 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added and boiled for 5 minutes to stop the reaction. The liberated reducing sugars (glucose equivalents) were estimated by glucose standard curve using the 3, 5- dinitrosalicylic acid (DNS) (Miller, 1959).The colour developed was read at 510 nm using a UV- spectrophotometer. Enzyme activity (IU) was calculated as the amount of enzyme releasing one µmol of glucose equivalents per minute under the assay conditions". The enzyme activities used for representations were the average values of three independent experiments.

$$\text{Enzyme activity (U/ml)} = \frac{\text{Amount of reducing sugars (X)} \times \text{Dilution factor}}{\text{Molecular weight of glucose} \times \text{time of incubation} \times \text{volume of enzyme}}$$

Evaluation of various agrobased substrates for production of alpha amylase by using Bacillus licheniformis

In an attempt to choose a potential substrate for solid state fermentation which supports amylase production, various agro residues like Wheat flour, Moong husk, Barley flour, Corn flour, Gram flour, Arhar husk, Mustard oil cake, Coconut oil cake, Rice bran, wheat bran, Potato peel, Sweet potato peel, Banana peel, Sugarcane baggase, Soybean husk were evaluated individually. SSF was carried out at 37°C for 48 h and crude enzyme was extracted by centrifugation at 8000 rpm for 10 minutes. The supernatant so obtained after centrifugation was used for enzyme assay. The substrate showing highest enzyme activity was considered potential substrate for production of alpha amylase

Optimization of fermentation process parameters for production of alpha amylase

Best substrate was employed for further optimization of production process parameters namely initial moisture content (55, 60, 65, 70, 75, 80, 85 and 90 %), incubation time (24, 48, 72, 96, and 120 h), incubation temperature (35, 40, 45, 50, and 55°C), initial pH (4, 5, 6, 7, 8, 9, and 10) of the medium, inoculums size (0.5, 1, 2, 4, 6, and 8 x 106 CFU/ml).

Optimization of medium components for production of alpha amylase in SSF

To study the efficacy of various inducers the fermentation medium was supplemented independently with 1% carbon sources (glucose, lactose, maltose and soluble starch), inorganic nitrogen sources (0.15M) (Ammonium nitrate,

Ammonium chloride and Ammonium sulphate), 1% organic nitrogen sources (peptone, tryptone, yeast extract, soybean meal) and phosphate (KH2PO4) concentration (0.01M, 0.02M, 0.03M and 0.04 M) and optimized for production of alpha amylase.

Results and Discussion

Evaluation of various agrobyproducts for the production of α- amylase by ***Bacillus licheniformis*** *under solid state fermentation*

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. Fifteen agrobased substrates namely wheat flour, Moong husk, barley flour, corn flour, gram flour, Arhar husk, Mustard oil cake, coconut oil cake, rice bran, wheat bran, potato peel, sweet potato peel, banana peel, sugarcane baggase, soybean husk were screened for alpha amylase production with reference strain Bacillus licheniformis MTCC 429 All the substrates supported the growth of Bacillus licheniformis and the enzyme formation by the culture, while wheat bran proved superior to the other substrates under Triton X- 100 extracting medium. A high titer of amylase (154.17 U/ml) was obtained in a medium containing wheat bran alone as the substrate. The suitability of other substrates for amylase production were as follows; sugarcane baggase (148.73U/ml), banana peels (152.47U/ml) and sweet potato peel(149.77U/ml) in Tween 80 medium (Table 1; Figure 1).

Table 1: Production of α- amylase by Bacillus licheniformis on various agro- substrate under SSF

S.No	Substrate	Enzyme activity(IU/ml/min)			
		Distilled water	Phosphate Buffer	Tween 80	Triton X-100
1.	Wheat flour	100.10	108.63	103.70	104.56
2.	Moong husk	65.89	64.90	61.40	66.10
3.	Barley flour	76.88	90.34	97.70	98.40
4.	Corn flour	79.12	61.27	76.86	84.78
5.	Gram flour	97.86	71.90	66.70	59.25
6.	Arhar husk	34.13	36.78	32.22	34.67
7.	Mustard oil cake	30.52	41.68	69.80	51.27
8.	Coconut oil cake	38.91	62.80	65.89	64.90
9.	Rice bran	72.76	129.45	139.66	117.34

10.	Wheat bran	102.33	119.48	150.54	154.17
11.	Potato peel	116.14	128.36	142.78	119.82
12.	Sweet potato peel	127.63	140.33	149.77	138.16
13.	Banana peel	115.39	147.28	152.47	133.26
14.	Sugarcane baggase	109.42	135.75	148.73	142.38
15.	Soybean husk	108.23	113.84	125.94	107.96

Substrate: F(cal) = 31.40 > F (tab) = 1.93 (S) at 5%

Extraction medium : F(cal) = 6.92 > F (tab) = 2.82 (S) at 5%

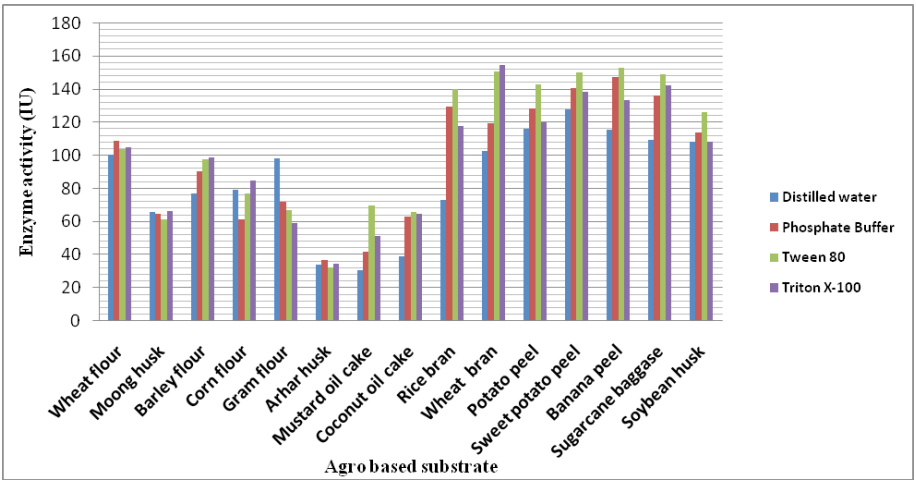

Figure 1: Production variation of α- amylase by Bacillus licheniformis from different agrobased substrates under SSF

In industries there is an essential need for an economical and simple indigenous system for producing high titers of alpha amylases. One alternative low cost and feasible production method is the use of SSF. However, there are several factors, which affect SSF processes. Among these, selection of a suitable strain, substrate and selection of process parameters are crucial (Pandey *et al.,* 2000). In the present investigation, SSF was applied for the production of alpha amylase by using *Bacillus licheniformis*. This study revealed high levels of α-amylase production with *Bacillus licheniformis* on sugarcane baggase and wheat bran as the solid substrates. In the present investigation wheat bran was found to be best for the production of α- amylase. The productivity levels of α- amylase from wheat bran were found to be high in other published reports of Babu and Satyanarayana (1995); Mamo and Gressesse (1999); Ramdas *et al.* (1996); Ramesh and Lonsane (1987).The suitability of a particular substrate in a SSF process for the production of α-amylase appears to be governed by the physicochemical requirements of

the microorganism used. This may be due to the fact that it contains sufficient nutrients and does not aggregate even in moist conditions, thus providing a large surface area (Ramesh and Losane, 1990). In a similar study conducted by Ashraf *et al***.** (2005) in which agricultural byproducts such as wheat bran, sunflower meal, soybean meal, cottonseed meal and rice bran were evaluated for the production of α- amylase by using *Bacillus licheniformis* GHB8 and found wheat bran to be the best substrate giving maximum yield. Similarly El Shishtawy *et al*. (2014) carried out production of α- amylase by *Bacillus megatherium* using different SSF sources such as wheat bran, grasses, palm leaves, date seeds and reported maximum production in case of wheat bran as compared to other agricultural residues. Tabassum *et al***.** (2014) used wheat bran, maize bran, maize starch for the production α- amylase and reported wheat bran (15%) the best carbon source to support maximum values of all kinetic parameters related to product formation. Niaz *et al*. (2010) studied the effect of different carbon sources such as wheat and maize bran for the biosynthesis of α- amylase by using *Bacillus licheniformis* RT_7YC strain and found maximum growth and amylase enzyme production in fermentation medium with wheat bran. Nagrajan *et al***.** (2010) screened two different agro-residues for the production of α- amylase by using *Bacillus subtilis* and obtained high amylase enzyme yield from wheat bran as compared to rice bran. Raj and Hemashenpagam (2012) carried out solid state fermentation of wheat bran and rice bran by using *Bacillus* sp. and reported maximum enzyme activity of α-amylase from wheat bran. Kalaiarasi and Parvatham (2013) also reported wheat bran as best substrate for the production of α- amylase among different substrates like wheat bran, millet bran, black gram bran, green gram bran and oil cakes like coconut, seasame, cottonseed, groundnut.

3.2 Optimization of process parameters for production of alpha amylase by using *Bacillus licheniformis* under SSF condition

3.2.1 Effect of inoculum sizes on alpha amylase production by Bacillus licheniformis

The inoculum level is an important factor for the production of amylase. High inoculum levels are inhibitory in nature. The present examination on production of α- amylase by solid state fermentation of wheat bran with varying inoculums sizes was conducted. The study showed the effect of inoculum size (ml) on α- amylase production by Bacillus licheniformis. The highest amylase enzyme production (178.84 IU) was obtained at an inoculum level of 6 ml (106 cfu/ml).There was a gradual increase of amylase enzyme production by increasing inoculum size from 0.5 to 6 ml while a further increase of inoculum showed a decrease in activity (Table 2; Figure 2).

Table 2. Production of α- amylase under different Inoculum sizes of Bacillus licheniformis

S.No	Inoculum size (106CFU/ml)	Enzyme activity(IU)
1.	0.5	78.58
2.	1	104.46
3.	2	153.95
4.	4	169.38
5.	6	178.84
6.	8	100.35

r =0.306, tcal =0.64<ttab =2.77 at 5% (NS), Y = 4.29 X + 115.51

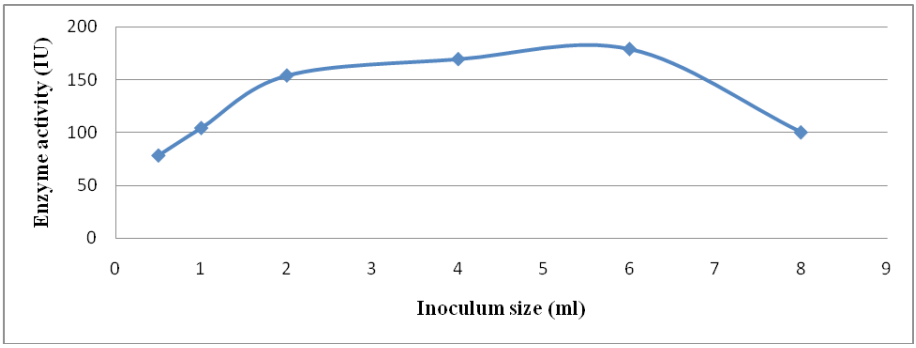

Figure 2. Alpha amylase production by Bacillus licheniformis under different inoculums sizes in SSF

3.2.2 Effect of different moisture content of SSF medium on alpha amylase production by Bacillus licheniformis

Moisture is one of the most important parameters in SSF that influence the organism and thereby enzyme production. Low and high moisture levels of the substrate affect the growth of the microorganism resulting in varying enzyme production. The present study conducted solid state fermentation of wheat bran by using Bacillus licheniformis under different moisture concentration. The maximum α-amylase production (171.89 IU) was obtained when the initial substrate moisture was 80%. A gradual increase in the production of α-amylase was observed with increase in moisture content from 55-80%. Further increase in moisture content of 5% resulted in a sharp decline of amylase enzyme production (168.64 IU) (Table 3 and Figure 3).

Table 3: Production of α- amylase by Bacillus licheniformis under different moisture content of SSF medium

S.No	Moisture content (%)	Enzyme activity(IU)
1.	55	105.61
2.	60	119.17
3.	65	128.83
4.	70	153.76
5.	75	160.74
6.	80	171.89
7.	85	168.64
8.	90	141.48

r =0.765, tcal =2.88<ttab =2.44 at 5% (NS), Y = 1.51 X + 34.22

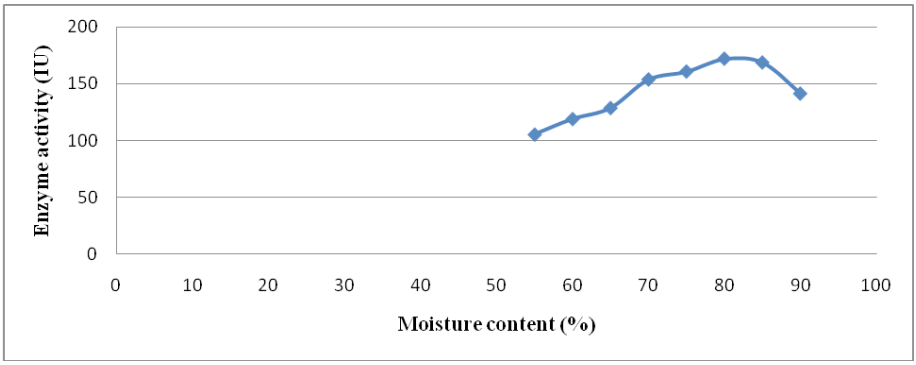

Figure 3. Variation of alpha amylase production by Bacillus licheniformis under different moisture content of SSF medium

The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. High moisture leads to reduction in substrate porosity, changes in the structure of the substrate particles and reduction of the gas volume that limits the oxygen transfer. Babu and Satyanarayana (1995) also achieved a marked improvement in enzyme production by optimizing moisture content in the medium when the moisture level was at the ratios of 1:1.5.Similar results were also demonstrated in this study, where a high amylase enzyme titer (171.89 IU) was recorded when the initial moisture level was 80%. A reduction in enzyme production at high initial moisture content may be due to a reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume. In addition, reduction in enzyme production is due to low moisture content that causes a reduction in the solubility of nutrient of the substrate and a low degree of swelling affecting the bacterial growth.

Rameshkumar and Sivasudha (2011) also reported maximum yield of α- amylase from rice bran at 80% moisture content by using Bacillus subtilis and obtained decreased level of yield at further increase in moisture content. El-Shishtawy et al. (2014) also reported the maximum α- amylase production from wheat bran at moisture content of 80%. Nandakumar et al. (1996) obtained optimum α-amylase production from wheat bran at 65% moisture content by using Bacillus coagulans. Rai and Solanki (2014) reported highest amylase enzyme yield at 80% while Tabassum et al. (2014) reported maximum production of α- amylase at 85% moisture content by using Bacillus licheniformis. Kalairasi and Parvatham (2013) obtained the maximum enzyme production with moisture in the ratio of 1:2. Nagrajan et al. (2010) reported maximum amylase enzyme yield from wheat bran at 88% moisture level by using Bacillus subtilis. Sameh et al. (2013) reported maximum enzyme production from crude millet at 86% moisture content. Noreen et al. (2002) reported highest α- amylase yield from banana peel at 84% moisture content while Ashraf et al. (2005) obtained best results from wheat bran at the moiture ratio 1:1 by using Bacillus licheniformis.

Effect of pH of SSF medium on alpha amylase production by Bacillus licheniformis

The initial pH of fermentation medium has a significant effect on bacterial growth and enzyme production. The present study conducted the effect of pH on alpha amylase enzyme production from wheat bran by Bacillus licheniformis. The production medium was adjusted with different pH ranging from 4 to 10. From the observation obtained in the present examination, maximum α-amylase production (170.34 IU) was recorded at pH of 6.0. There was a 10.5% (152.46 IU) and 33.8% (112.75 IU) decrease in activities at pH 7.0 and pH 8.0, respectively (Table 4 and Fig 4).

Table 4. Production of alpha amylase by Bacillus licheniformis under different pH of SSF medium

S.No	pH	Enzyme activity(IU)
1.	4	129.44
2.	5	142.85
3.	6	170.34
4.	7	152.46
5.	8	112.75
6.	9	81.14
7.	10	49.33

r =-0.767, tcal =-2.67 >ttab =2.57 at 5% (S), Y = 225.09 - 15.04 X

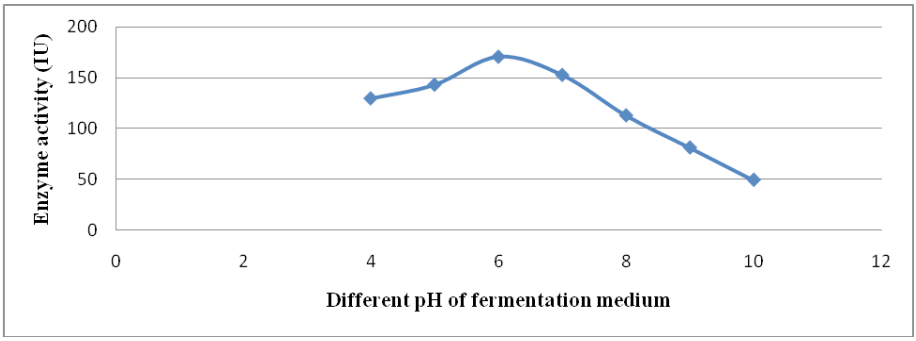

Figure 4: Variation in alpha amylase production by Bacillus licheniformis due to different pH of SSF medium

pH is one of the important factors that determine the growth and enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. As the metabolic activities of the microorganism are very sensitive to changes in pH, alpha amylase production was found to be affected when the pH level is above or below the optimum pH. Kalairasi and Parvatham (2013) also reported high enzyme titre when the pH of the medium was maintained at 7. Nagrajan et al. (2010) reported maximum amylase production by using Bacillus subtilis when the initial medium was neutral (pH=7) for both wheat bran and rice bran. Noreen et al. (2002) obtained maximum yield of alpha amylase enzyme from banana peels under the SSF condition by using Bacillus subtilis at pH 7. Ashraf et al. (2005) observed maximum production of alpha amylase by using Bacillus licheniformis. The culture (GHB8) gave the maximum production of alpha amylase at pH 7.5 when the wheat was replaced with cottonseed meal (3:1). Varalakshmi et al. (2012) reported pH 7.5 for the optimal enzyme production by using Pseudomonas sp. under solid state fermentation. Serin et al. (2012) also found the maximum enzyme production from rice bran by using Bacillus circulans ATCC 4516 when the initial pH was 7.5. Furthermore increase in the pH resulted in decrease of enzyme production. Niaz et al. (2010) also produced α- amylase from (10%) wheat bran by using Bacillus licheniformis RT7YC strain at pH 7.0. Hassan and Karim (2012) produced alpha amylase enzyme from oil palm fruit bunch under solid state fermentation at pH 7.0. Raj and Hemashenpagam (2012) also found maximum enzyme production at pH 7.0 under SSF condition by using Bacillus. The growth and production of alpha amylases requires nearly neutral pH i.e. ranges from pH 6-8 (Sivaramakrishnan et al., 2006; Regulapati et al., 2007). El-Shishtawy et al. (2014) observed amylase production from wheat bran at pH 5 by using Bacillus sp. Rai and Solanki (2014) found amylase production from the mixture of wheat bran and barley bran by using B. amyloliquefaciens at pH 7.0. Tabassum et al. (2014) reported maximum level of alpha amylase production from wheat bran using Bacillus licheniformis RTPE-1 strain at pH 7.0.

Effect of Incubation temperature on alpha amylase production by Bacillus licheniformis

Normally the incubation temperature for the alpha amylase production is much related to the growth of the microorganism. Therefore the optimum incubation temperature for the fermentation depends on whether the microorganism is mesophilic or thermophilic. The bacterial alpha amylase is produced at much wider range of temperature. In the present study alpha amylase produced by Bacillus licheniformis showed considerable enzyme activities in the range of 35°C - 55°C. The enzyme activity increased upto 40°C (168.78 IU) and then the enzyme activity dropped tremendously at 50°C and 55°C. The optimal temperature for maximum amylase production (168.78 IU) was found to be 40°C (Table 5; Fig 5).

Table 5. Alpha amylase production by Bacillus licheniformis under different incubation temperature of SSF medium

S.No	Incubation Temperature(oC)	Enzyme activity(IU)
1.	35	149.62
2.	40	168.78
3.	45	132.45
4.	50	99.32
5.	55	90.56

r =-0.89, tcal =-3.5>ttab =3.18 at 5% (S), Y = 296.96 - 3.75 X

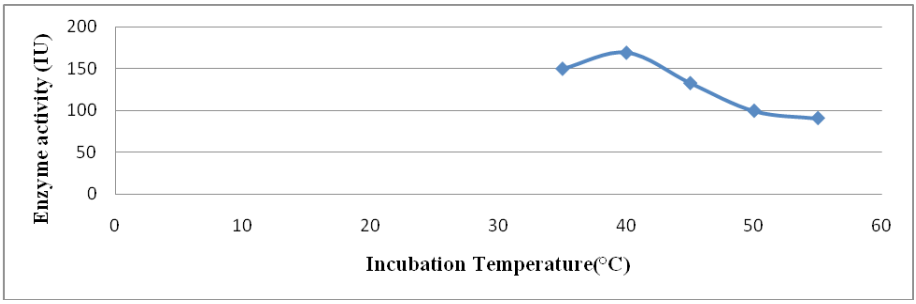

Figure 5: Variation of alpha amylase production by Bacillus licheniformis under different Incubation temperature of SSF

Similar results have been reported by Serin et al. (2012) for the production of alpha amylase when rice bran was used with B. circulans at 37°C. Raj and Hemashenpagam (2012) obtained maximum production of amylase at 55°C by using Bacillus sp. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial

activity and therefore the initial temperature is vital (Gangadharan et al., 2006). Temperature plays a significant role in development of the biological processes as it influences protein denaturation, enzyme inhibition and cell growth (Balkan et al., 2005). Varalakshmi et al. (2012) also reported 37°C for the production of enzyme when Pseudomonas sp. 2 was used with wheat bran and further increase of temperature decreased the enzyme production. Similar results were also found by Ashraf et al. (2005) when Bacillus licheniformis was used with wheat bran at 40°C. Further increase in the incubation temperature there was gradual reduction in the enzyme formation. Sameh et al. (2013) reported maximum enzyme production at 37°C when crude millet was used as substrate with a new Bacillus sp. UEB-S under solid state fermentation. Nagrajan et al. (2010) reported similar results when wheat bran and rice bran were fermented with Bacillussubtilis. Kalairasi and Paravtham (2013) obtained optimal temperature for maximum alpha amylase production at higher temperature i.e. 50°C when Bacillus cereus was used with wheat bran. Temperature above 50°C resulted in lesser growth of Bacillus cereus and decreased the enzyme production. Moisture loss in solid substrate due to increase in temperature might be the cause for lesser production of alpha amylase. Tabassum et al. (2014) reported the enzyme production at 37°C when wheat bran was used as substrate with Bacilluslicheniformis RTPE1. Rai and Solanki (2014) also reported 38°C fot maximum yield of enzyme by using Bacillusamyloliquefaciens with a mixture of wheat bran and barley bran (1:1). El- Shishtawy et al. (2014) observed optimum temperature to be 45°C for amylase production from wheat bran by using the Bacillus sp. In other study the optimum temperature recorded for maximum growth and alpha amylase production by B. subtilis was 35°C (Unakal et al., 2012).

Effect of different Incubation periods on alpha amylase production by Bacillus licheniformis under SSF condition

In SSF, the incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The different incubation times selected for α- amylase production were 24, 48, 72, 96 and 120 h. There was a decreasing trend in enzyme production from 48 to 120 h of incubation. Bacillus licheniformis produced high titers of enzyme (155.06 IU) at 48 h of incubation. A steady decline in enzyme activity was observed after 48 h to 120 h, with the lowest enzyme titer (83.56 IU) at 120 h (Table 6; Fig 6).

Table 6. Alpha amylase production by Bacillus licheniformis under different incubation periods

S.No	Incubation Time(hr)	Enzyme activity(IU)
1.	24	119.39
2.	48	155.06
3.	72	145.62
4.	96	100.16
5.	120	83.56

r =-0.66, tcal = - 1.53>ttab =3.18 at 5% (NS), Y = 158.72 - 0.527 X

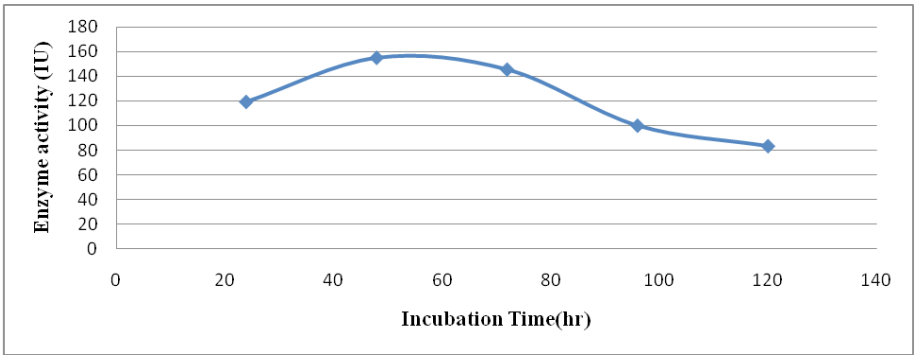

Figure 6. Variation of alpha amylase production by Bacillus licheniformis under different Incubation periods

The investigation showed variation in the alpha amylase production with different fermentation periods and revealed that maximum amylase was produced at 48 h incubation period. In a similar study presented by Rameshkumar and Shivasudha (2011) SSF was carried out on rice bran with initial moisture content of 75% at 37°C, they reported maximum alpha amylase enzyme production after 44 h. The reason for this might be due to the denaturation of the enzyme caused by the interaction with other components in the medium. It could have been also be due to the fact that the microorganism might be on its decline phase during the second day of fermentation and resulted in the decreased enzyme production. The present report is also in agreement with Serine et al. (2012) who reported high production of α- amylase from rice bran using Bacillus circulans ATCC 4516 at 48 h. The production of enzyme decreased after 48 hours. Enzyme production is related to the growth of the microorganism. Growth of the organism would have reached a stage due to insufficient nutrients that indirectly stimulates production of secondary metabolites. Ashraf et al. (2005) also obtained maximum enzyme production after 48 h of inoculation from SSF of wheat bran by using

Bacillus licheniformis. Hassan and Karim (2012) observed optimum incubation time with the highest amylase activities for both substrates namely rice straw and OPEFB fibres after 48 h and a further increase in incubation time led to a significant reduction in enzyme activity. The decline in enzyme activity might be due to denaturation of α-amylase as a result of interactions with compounds in the fermentation medium (Ramesh and Losane, 1987). Another possible reason is due to the presence of some inhibitors that might inhibit the enzyme secretion. Raj and Hemashenpagam (2012) also reported 48 h of incubation for the highest amylase enzyme production from wheat bran by using Bacillus sp. under solid state fermentation. Sameh et al. (2013) reported four days incubation for maximum amylase enzyme production by using Bacillus sp. from crude millet. Nagrajan et al. (2010) also reported 40h of incubation for maximum amylase enzyme production from wheat bran by using Bacillus sp. while decreased yield was observed due to further increase of incubation time. In contrast to the present study Kalairasi and Parvatham (2013), Babu and Satyanarayana (1995) and Anto et al. (2006) have reported that 72 h incubation for good enzyme production with Bacillus cereus MTCC 10202, Bacillus coagulans and Bacillus cereus. Baysal et al. (2003) also reported 48 h as optimum incubation time for alpha amylase production using Bacillus sbutilis. In most cases the optimum incubation period for alpha amylase production under SSF using Bacillus sp. varied from 48 to 72 h depending on the environmental conditions (Sivramakrishnan et al., 2006). Rai and Solanki (2014) and Tabassum et al. (2014) have also reported 72 h incubation time for Bacillus amyloliquefaciens and Bacillus licheniformis which is in contrast with the present study. Rameshkumar and Sivasudha (2011) have reported similar result of maximum enzyme production after 48 h of incubation and decrease in the enzyme yield on further increase in incubation time. El-Shishtawy et al. (2014) have reported maximum amylase enzyme production after 24 h of incubation time using Bacillus sp. grown on wheat bran. α- Amylase production by Bacillus sp. in SSF showed that the organism colonized well on the solid substrate and exhibited good growth after 24h at 37°C. Maximum enzyme production was achieved after 48 h incubation (Sodhi et al., 2005).

Optimization of Nutritional Parameters for α- amylase production using Bacillus licheniformis under solid state fermentation

Effect of additional carbon sources on α- amylase production by Bacillus licheniformis under SSF condition

Growth and enzyme production of any organism are greatly influenced by the nutrient available in the growth medium. Alpha amylase is an inducible enzyme. The carbon sources in the medium are found to exert a profound effect on the

enzyme production behavior. Some carbon source supports good growth with low enzyme production while other support good growth as well as enzyme secretion. The present investigation was conducted to optimize fermentation medium with carbon sources for industrial enzyme production. The experiment was conducted on the production medium containing wheat bran and supplementation with different carbon sources namely glucose, lactose, maltose, soluble starch at 1% (w/v) concentration. The production medium was then fermented using Bacillus licheniformis for the production of α- amylase. In the present study there was a significant increase of amylase enzyme yield in media supplemented with maltose (178.46 IU) whereas the production of α- amylase by B. licheniformis was suppressed when bacterium was grown in the production medium with glucose (142.44 IU) and lactose (148.68 IU) (Table 7;Fig 7).

Table 7. Production of alpha amylase by Bacillus licheniformis with additional carbon sources supplemented to SSF

S.No	Carbon source	Enzyme activity(IU)
1.	Control	156.18
2.	Glucose (1%)	142.44
3.	Lactose (1%)	148.68
4.	Maltose (1%)	178.46
5.	Soluble starch (1%)	166.26

Fcal =606.48 > Ftab =11.25 at 1% (S)

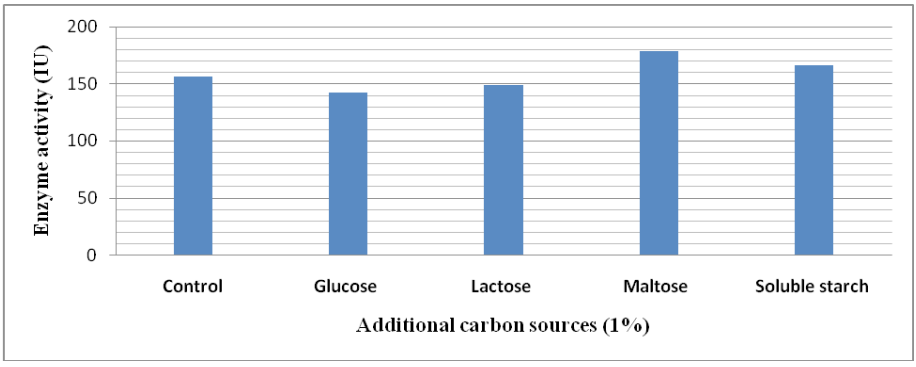

Figure 7. Variation in α- amylase production by Bacillus licheniformis with additional carbon sources supplementation

Similar result was also recorded by Babu and Satyanarayana (1995) where glucose did not enhance α- amylase production by Bacillus coagulans under solid state fermentation using wheat bran. Lactose exhibited repressive effect on enzyme

production. Rama and Srivastava (1995) had reported that easily metabolizable carbohydrate might result in the better growth of the bacteria along with reduction in enzyme formation. Ashraf et al. (2003) also reported the lower rate of enzyme production when glucose and lactose were supplemented into the medium which is in agreement with the present study. The high starch concentration promoted high enzyme formation because the organism used the starch much slowly than other sugars for its respiratory activity. Hence it can be speculated that the slow metabolic rate favours amylase production. Ramesh and Losane (1990) also found the soluble starch as best carbon source for α- amylase production by Bacillus licheniformis M27. On the contrary Hassan and Karim (2012) obtained increased α- amylase production using Bacillus subtilis from rice straw by increasing the glucose concentration in the medium. These results may infer that different bacillus sp. require different carbon source for the synthesis of α- amylase. In another study conducted by Nagrajan et al. (2010) higher yield of α-amylase using Bacillus subtilis was obtained from wheat bran medium supplemented with 0.1% glucose and sucrose.Kalairasi and Parvatham (2013) also fermented different carbon supplements (starch and maltose) into the wheat bran medium under SSF by using Bacillus cereus MTCC 10202 and reported high enzyme titre with starch followed by maltose. Glucose supplementation resulted in the repression of enzyme production. This might be due to the feedback inhibition caused by the presence of glucose as reported by Rama and Srivastava (1995). Sindhu et al. (2009) and Kunamneni et al. (2005) also reported similar results in which alpha amylase production was induced by starch and its hydrolytic product maltose. Glucose has inhibitory effect on production and acts as catabolite repressor as reported by Nandakumar et al. (1996) and Alva et al. (2007). Ramesh Kumar and Sivasudha (2011) found starch as best carbon source supplementation with rice bran for α- amylase production when Bacillus subtilis MAFE118079 was used as inoculum. Rai and Solanki (2014) also reported maltose (1%) as best carbon source supplementation for enzyme production when Bacillus amyloliquefaciens was used with agricultural residue mixture of wheat bran and barley bran (1:1). El-Shishtawy et al. (2014) also reported increased α- amylase production by maltose and starch supplementation and decreased production by lactose supplementation by using Bacillus sp. Sarethy et al. (2012) also found 1% starch as best for inducing α- amylase production. In a study reported by Pandey et al. (2000), higher cell density and higher specific growth rate were obtained from glucose but higher enzyme activity and higher specific enzyme activity were obtained from starch. Nguyen et al. (2000) also found that maltodextran was the best carbon source for α-amylase production.

Effect of additional Organic Nitrogen sources for the alpha amylase production by Bacillus licheniformis

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes in SSF system. Addition of various organic nitrogen sources namely peptone, tryptone, yeast extract, soy peptone to the medium was investigated in the present study. In comparison with control there was significant increase in enzyme yield in case of the supplementation of soy peptone. Among the various organic nitrogen sources tested soy peptone at 1% showed maximum alpha amylase yield (171.68 IU) followed by yeast extract (156.81 IU).Of the nitrogen sources tested, soy peptone was the best inducer for alpha amylase production while tryptone was a poor inducer. Peptone suppressed the amylase activity (147.74 IU) (Table 8; Fig 8).

Table 8. Production of alpha amylase by Bacillus licheniformis due to additional Organic Nitrogen sources supplementation

S.No	Nitrogen source	Enzyme activity(IU)
1.	Control	152.98
2.	Peptone (1%)	147.74
3.	Soy Peptone (1%)	171.68
4.	Yeast extract (1%)	156.81
5.	Tryptone (1%)	133.34

Fcal =592.24 < Ftab =11.25 at 1%, (S)

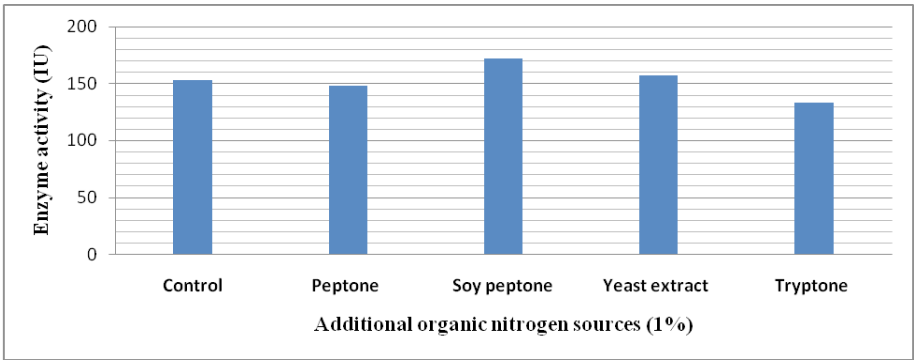

Figure 8. Variation in alpha amylase production by Bacillus licheniformis due to additional Organic Nitrogen sources supplementation

The influence of yeast extract on α- amylase production might be due to the presence of vitamin B- group, free amino acids and carbohydrates. Kalairasi and

Paravatham (2013) obtained similar observations in which maximum α- amylase production was produced by yeast extract followed by peptonewhich might be due to changes in C/N ratio.The observed results are also in concurrence with the work reported by Ashgar et al. (2007) for Bacillus subtilis JS2004 who have reported yeast extract as the best nitrogen supplement for α- amylase production.The nitrogen is metabolized to produce primary amino acids, nucleic acids, proteins and cellular components. Low levels of nitrogen are inadequate for enzyme production whereas excess nitrogen can equally be detrimental causing a complete inhibition of enzyme production.Hassan and Karim (2012) also observed similar effect when yeast extract was supplemented as the additional nitrogen source. El-Shishtawy et al. (2014) also reported induced α- amylase production by adding yeast extract.Similar observation was also reported by Aiyer (2004), where supplementation of peptone has an influence on α- amylase production by Bacillus licheniformis SPT 278. This finding is also supported by Bhargava et al. (2008) that nutrient supplementation from organic sources increased enzyme production to a greater extent.Yeast extract is undefined media which contain high nutritional amino acids and compatible nitrogen and carbon sources to support the growth of Bacillus subtilis. Noreen et al. (2002) have reported that Bacillus subtilis produced increased yield of α- amylase by supplementing 0.1% yeast extract. Rameshkumar and Sivasudha (2011) observed enhanced amylase production by Bacillus subtilis MAFE 118079 when 1% peptone as nitrogen source was added additionally into the medium.

Table 9: Production of alpha amylase by Bacillus licheniformis due to additional Inorganic nitrogen sources supplementation

S.No	Inorganic Nitrogen source	Enzyme activity(IU)
1.	Control	155.10
2.	Ammonium nitrate(0.15M)	132.65
3.	Sodium nitrate(0.15M)	121.42
4.	Ammonium chloride(0.15M)	172.36
5.	Ammonium Sulphate (0.15M)	144.84

Fcal =270.33 > Ftab =11.25 at 1% (S)

Effect of additional Inorganic nitrogen sources supplemented on alpha amylase production by Bacillus licheniformis under SSF condition

Various inorganic nitrogen sources namely ammoinium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate were investigated for the production α- amylase by using Bacilluslicheniformis. Among all the inorganic sources, maximum enzyme production was recorded due to ammonium chloride at 0.15M

supplementation into the medium. Ammonium sulphate, sodium nitrate and ammonium nitrate were found to be inhibitory in amylase enzyme production (Table 9; Figure 9).

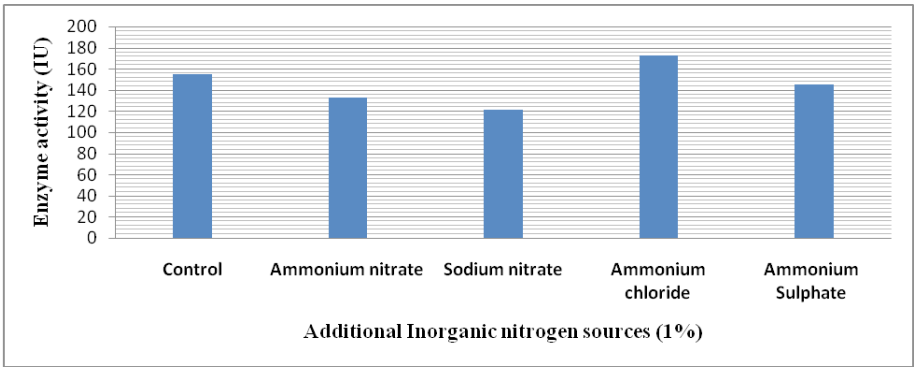

Figure 9. Variation in alpha amylase production by Bacillus licheniformis due to additional Inorganic Nitrogen sources supplementation

Addition of sodium nitrate to the wheat bran showing negative influence on the production of α- amylase was also reported by Hassan and Karim (2012). The repressing production of α- amylase is because of the nitrate (NO3-) compound that is more difficult to be degraded into a simpler compound of nitrite (NO2-) and subsequently into ammonium. As in ammonium chloride the nitrogen source is readily utilized by Bacillus licheniformis. Thus the consumption of nitrogen sources in ammonium chloride and sulphate is faster and more effective than that in sodium nitrate.

Effect of different concentration of Phosphate on Alpha amylase production by Bacillus licheniformis under SSF condition

Table 10: Production of alpha amylase by Bacillus licheniformis due to varying concentration of Phosphate supplementation

S.No	Phosphate concentration	Enzyme activity(IU)
1.	Control	152.88
2.	0.01M	164.78
3.	0.02M	157.63
4.	0.03M	154.65
5.	0.04M	159.43

r =0.101, tcal =0.175<ttab =3.18 at 5% (NS), Y = 29.7 X + 157.28

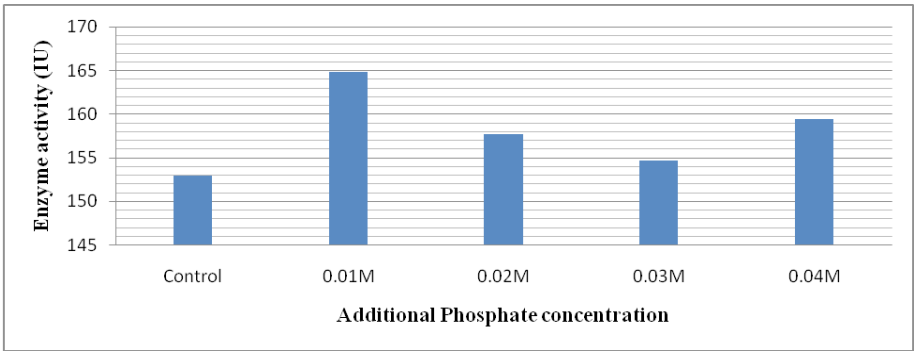

Figure 10. Variation in alpha amylase production by Bacillus licheniformis due to varying concentration of Phosphate supplementation

Statistical Analysis

The obtained data were analyzed using ANOVA and Correlation.

Conclusion

Commercially most of the production of α – amylase is carried out in submerged fermentation but solid – state fermentation can be looked as a potential tool for its production, especially applying agro industrial residues as substrate. Based on the Results of this investigation, it can be concluded that wheat bran has higher efficiency in α-amylase production among the agroresidues using Bacillus licheniformis.

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