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Full Length Research Paper

Optimization of culture parameters to enhance production of amylase and protease from *Aspergillus awamori* in a single fermentation

Sangeeta Negi[1]* and Rintu Banerjee[2]

[1]Biotechnology Section, Applied Mechanics Department, Motilal Nehru National Institute of Technology, 221 004 Teliarganj, Allahabad, India.

[2]Agriculture and Food Engineering Department, Microbial Biotechnology, 721302 Kharagpur, India.

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Concomitant production of amylase and protease by *Aspergillus awamori* nakazawa MTCC 6652 was enhanced in a single fermentation by media engineering and optimization of other important parameters. Wheat bran was considered as a suitable substrate for production of amylase and protease by *A. awamori* in a single fermentation as none of the additional substrate such as powder of peanut, corn, soybean seeds and sunflower seeds was effective in enhancing the concurrent production of enzymes further. Optimum amylase yield of 4528.4 ± 121U/gds and protease yield of 250.4 ± 10 U/gds was achieved with wheat bran to Czapek-dox ratio of 1:1.5 (w/v), 96 h incubation, 35˚C temperature, pH 5.5 and 85% relative humidity. Media engineered with 1% casein and 1% starch solution increased yield of amylase and protease by 2.07 fold (9386.5 ± 101 U/gds) and 3.73 fold (934.8 ± 67 U/gds), respectively, therefore, considered as the most suitable media for concomitant production of amylase and protease by *A. awamori* in a single fermentation.

Key words: Amylase, protease, solid state fermentation, optimization.

INTRODUCTION

Amylolytic enzymes (alpha-amylase E.C. 3.2.1.1, beta-amylase 3.2.1.2 and glucoamylase E.C. 3.2.1.3) and proteases (acid and alkaline proteases) are among the most important industrial enzymes because of their industrial applications in the field of brewing, textile, paper, sugar, distilling, food processing, pharmaceuticals, tannery, waste bio-degradation, detergent etc. (Pandey et al., 2000), concomitant production of these enzymes in a single fermentation is the current need of the enzyme industry.

Selection of a suitable strain capable of producing both these enzymes concomitantly with commercially acceptable yield was a crucial step. After detail study of the relevant literature decision was taken to explore *Aspergillus awamori* for multienzymes production. *A. awamori* has been reported to be very effective for production of amylases and proteases, although not much has been reported on concomitant production of multi-enzymes, Pandey et al. (2000), Peixoto et al. (2003), Sumantha et al. (2006) and Yamamoto et al. (2005).

Conventionally, multienzymes are produced by mixing various enzymes directly through genetically engineered microbes endowed with multi-functions (Zaghloul et al., 2000) and by mixed culture consisting of different well-designed microbes, Zhang et al. (2004). These methods have their own disadvantages; hence, appropriate bioprocesses with a well-established bioengineering were needed to be developed for efficient and simultaneous production of amylase and protease by a single microbial culture in a single fermentation.

Present investigation focused on two important aspects; optimization of parameters and effects of various additives, for achieving optimum production of amylase and protease in a single fermentation by *A.awamori*. Wheat bran was selected as substrate because it has been reported as the most common and effective substrate,

*Corresponding author. Email: sn5@mnnit.ac.in. Tel: +919415015811. Fax: +91-532-2271200.

Pandey et al. (1999). Optimum utilization of environmental conditions, such as incubation period, temperature, pH, relative humidity etc. was carried out for achieving high yield. Additives play an important role in fermentation processes; hence, investigation was also carried out on the effects of various additives such as nitrogen sources, carbon sources, metal ions, surfactants and hormones on the production of amylase and protease in a single fermentation.

MATERIALS AND METHODS

Chemicals

The chemicals used during this work were of analytical grade.

Microorganism

A. awamori nakazawa MTCC 6652 used for the present investigation was maintained on 2% malt-extract agar slants and stored at 4°C. A spore suspension was made by adding 25 ml of sterile distilled water to a 5 days old slant and scraped aseptically with inoculating loop. A suspension, having spore concentration of about 1.3×10^7 cells ml^{-1}, was used as inoculum for the subsequent fermentation.

Selection of substrate

Wheat bran was used as suitable substrate for the present investigation because its composition (15.5% protein, 64.51% carbohydrates, 4.2% fat and other nutrients also in desirable amount) was found appropriate for the production of desired enzymes.

Substrates manoeuvring

In order to enhance the simultaneous production of amylase and protease other substrates such as peanut, maize, soybean and sunflower seeds powder (grinded in mixer-grinder for 5 min) were supplemented with wheat bran in 1% (w/w) to enrich the media. Fermentation was carried out for 5 days at 35°C and other fermentation conditions were kept constant. After 120 h of incubation, fermented broth was filtered and centrifuged. Effects of various combinations of substrate with wheat bran as base were studied in triplicates and combination giving optimum concomitant yields of enzymes was selected as substrate for subsequent experiments.

Fermentation process

Fermentation was carried out in a Erlenmeyer flask (500 ml) containing 10.0 g of wheat bran soaked in 15 ml of salt solution (Czapek-dox: $NaNO_3$ 2.5 g/L, KH_2PO_4 1.0 g/L, $MgSO_4.7H_2O$ 0.5 g/L, KCl 0.5 g/L), shaked till entire substrate got moistened with Czapek-dox solution and then autoclaved. This mixture was inoculated with 2 ml of spores suspension aseptically and incubated for 4 days at 35°C and 85% relative humidity. Fermentation was conducted under various experimental conditions**.**

Extraction process

Fermented mass was soaked for 2 hrs in distilled water (in a ratio of 1:4) and squeezed with cheese cloth. The extract was centrifuged at 10,000 rpm for 15 min to remove the spores and other insoluble materials.

Determination of amylase activity

Amylase activity was measured following the method described by Bernfeld (1955). A reaction mixture containing 0.5 ml of 1% soluble starch solution prepared in 0.2 M acetate buffer and 0.5 ml of diluted enzyme solution was incubated at 50°C. After 10 min of incubation the reaction was terminated by adding 1.0 ml of DNS solution (1 g of DNS dissolved in 20 ml of 2 M NaOH, to which 30 g of sodium potassium tartarate and water were added to make it 100 ml). Reaction mixtures were boiled for 15 min and after cooling 18 ml water was added. Absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 micromole of glucose as reducing sugar in 1 min under the assay conditions.

Determination of protease activity

Protease activity was determined by caseinolytic method described by Walter (1984)**.** 0.05 ml of enzyme solution was added to 2 ml of casein solution with 0.65 ml of 0.2 M glycine NaOH buffer and incubated at 37°C for 20 min. Reaction was terminated by adding 0.2 ml 1 N HCl and unhydrolyzed casein was precipitated with 5 ml of 5% TCA solution. Thereafter, clear solution was separated by centrifugation at 9780 xg for 10 min and peptide fragment were measured. One unit of enzyme activity was defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of Bovine Serum Albumin (BSA) under the assay conditions.

Optimization of various parameters

Optimization of various parameters was carried out with “One at a time” strategy keeping all other variables constant except one. The variable under study was varied over a desired range in triplicates and optimal level was used for further investigation.

Preparation of engineered media

A mixture of starch and casein 1% (w/v) was prepared in Czapek-Dox in media and pure culture of *A.awamori* was inoculated to it. After 4 days of incubation at 35°C a clear film of spores could be seen over liquid media. This broth was vortexed and mycelia were removed to make a clear spore suspension.

Effects of additives

Different additives, such as carbon sources (dextrose, starch, sago, arrowroot, galactose and lactose in 1% w/w ratio), organic nitrogen sources (urea, yeast extract, casein and peptone in 1% w/w ratio), inorganic nitrogen sources (NH_4NO_3, $(NH_4)2SO_4$, NH_4Cl, KNO_3 and $NaNO_3$ in 1% w/w ratio), metal ions (0.05 mg/g $CaCl_2$, $MgCl_2$, $SnCl_2$, $FeCl_3$, $CoCl_2$ and $HgCl_2$), surfactants (0.05 mg/g SLS, Triton X-100, Tween 80 and 40) and hormones (0.0025 mg/g IAA, IBA, 2,4D and NAA) were added to fermentation media and their effects on enzymes production were studied by carrying out fermentation process in triplicates.

RESULTS

Effects of additional substrate

Additional substrates, such as powder of peanut, corn,

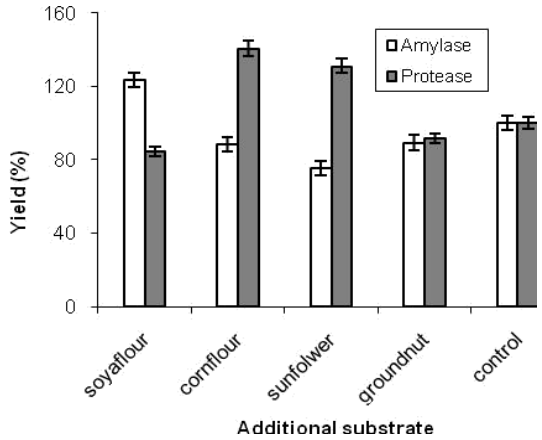

Figure 1. Effect of additional substrate on the production of amylase and protease.

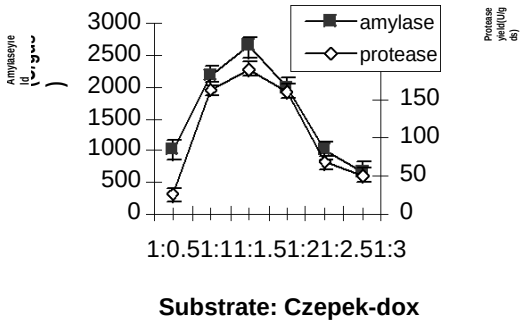

Figure 2. Effect of substrate to Czapek-dox ratio on the production of amylase and protease.

Table 1. Effect of incubation period on the production of amylase and protease.

Incubation period (h)	Amylase (U/gds)	protease (U/gds)
24	946.5 ± 38	21.5 ± 5
48	1797.3 ± 56	77.5 ± 7
72	3060.5 ± 77	146.5 ± 14
96	706.2 ± 87	256.4 ± 25
120	3529.5 ± 85	264.5 ± 32
144	2022.5 ± 75	154.5 ± 23

soybean seeds and sunflower seeds were supplemented with wheat bran in 1% (w/w) to enrich the media. Addition of none of the additional substrates was found effective in enhancing the yield of amylase as well as protease simultaneously (Figure 1). Soy flour increased amylase yield (1.23 fold) only, whereas corn (1.4 fold) and sunflower (1.3 fold) increased protease yield but reduced amylase yield.

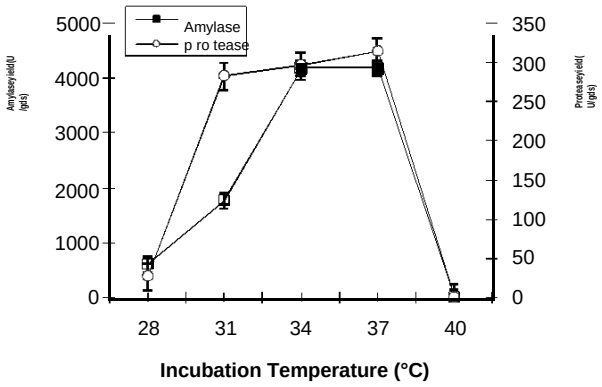

Figure 3. Effect of incubation temperature on the production of amylase and protease.

Optimization of solid to liquid ratio

In order to optimize the liquid-solid proportion, volume of Czapek-dox media was varied from 5 to 40 ml and substrate amount was kept constant at 10 g. As shown in Figure 2, substrate to liquid ratio of 1:1.5 (w/v) gave optimum yield of amylase and protease concomitantly.

Optimization of incubation period

The incubation period was varied from 24 to 144 h. The results in Table 1 showed that production of amylase increased with the increase in incubation period up to 96 h and beyond this there was no remarkable change in the yield of amylase. Yield of protease was also high at 96 h incubation. Its maximum value was at 120 h. Therefore, 96 h incubation was most suitable for optimum concomitant production of both enzymes.

Optimization of incubation temperature

Incubation temperature was varied from 28 to 40°C, keeping all other parameters constant. As shown in Figure 3, yields of amylase and protease were highest at 35 and 37°C, respectively. However, protease yield was also high at 35°C, so further fermentation was carried out at 35°C.

Selection of pH of media

The results in Table 2 showed that the yield of extracellular amylase and protease had been sensitive to initial pH of the medium. Amylase and protease productions were maximum at pH 5.5 and 4.5, respectively. However, further experiments were performed at pH 5.5 as yields of both enzymes were high at this level.

Table 2. Effect of initial pH of the media on amylase and protease activities.

Initial pH	Amylase activity (U/ml)	Protease activity (U/ml)
3.5	3102.9 ± 85	240.6 ± 21
4.5	4128.3 ± 101	309.2 ± 25
5.5	4528.4 ± 121	250.4 ± 10
6.5	4028.4 ± 96	201.5 ± 27
7.5	3521.5 ± 56	185.4 ± 25
8.5	2851.3 ± 45	108.5 ± 32
9.5	1493.2 ± 37	50.5 ± 18

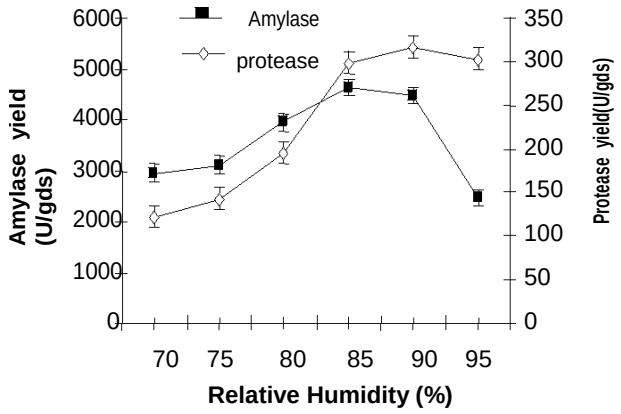

Figure 4. Effect of relative humidity on the production of amylase and protease.

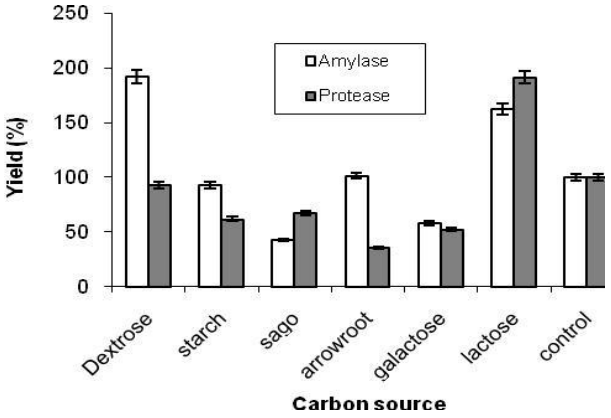

Figure 5. Effect of carbon sources on the production of amylase and protease.

Optimization of humidity

Relative humidity was varied from 70 to 95% keeping other parameters constant. Maximum production of amylase and protease was achieved at 85 and 90% relative humidity, respectively (Figure 4). Further investigation was carried out at 85% relative humidity as yields of both enzymes were high and stable at that level.

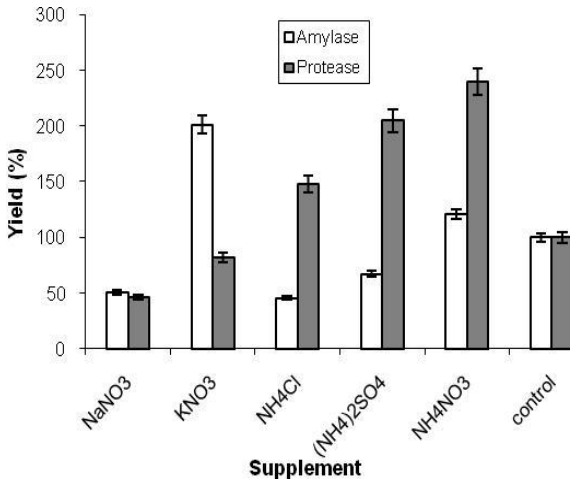

Figure 6. Effect of inorganic nitrogen sources on the production of amylase and protease.

Effect of additional carbon supplements

Only lactose increased production of amylase as well as protease by 1.62 and 1.91 fold, respectively (Figure 5). All other supplements either reduced or had no impact on yields of enzymes, except dextrose which increased amylase yield by 1.92 fold but it reduced protease activity.

Effect of additional nitrogen sources

Mycelia growth had been found very high in presence of inorganic nitrogen sources. Only NH_4NO_3 acted as enhancer for production of amylase and protease by 1.21 and 2.1 fold, respectively. Among other inorganic sources $(NH_4)_2SO_4$ and NH_4Cl increased protease production by 2.05 and 1.48 fold, respectively, but both led to reduction in amylase production (Figure 6). Other inorganic supplements had negative impact on production of both the enzymes.

Among organic supplements only peptone increased amylase and protease yield by 2.01 and 1.35 fold, respectively (Figure 7).

Effect of metal ions

Among metal ions only $HgCl_2$ stimulated the production of amylase as well as protease, by 2.44 and 2.62 folds, respectively (Figure 8). $FeCl_3$ adversely affected production

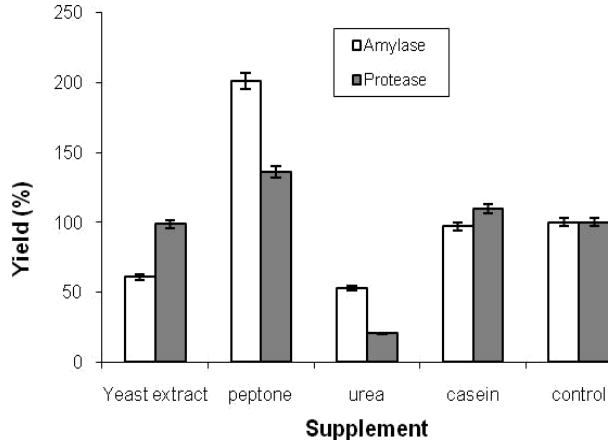

Figure 7. Effect of organic supplements on the production of amylase and protease.

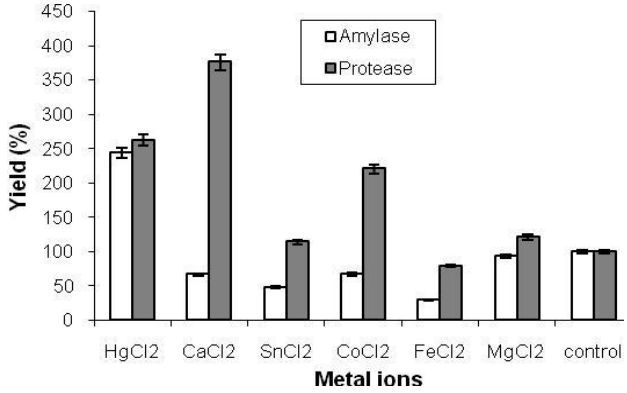

Figure 8. Effect of metal ions on the production of amylase and protease.

production of both the enzymes. All other metal ions enhanced protease production but remarkably reduced amylase production.

Effect of surfactants

Triton-X100 acted as stimulator for production of both amylase and protease, albeit marginally (Figure 9). Sodium laurate sulphate (SLS) increased amylase production by 1.28 fold but reduced protease production. Other non ionic surfactants showed negative influence on production of both the enzymes.

Effect of hormones

Indol acetic acid (IAA) and Indol-3-butyric acid (IBA) stimulated production of both the enzymes. Napthelene

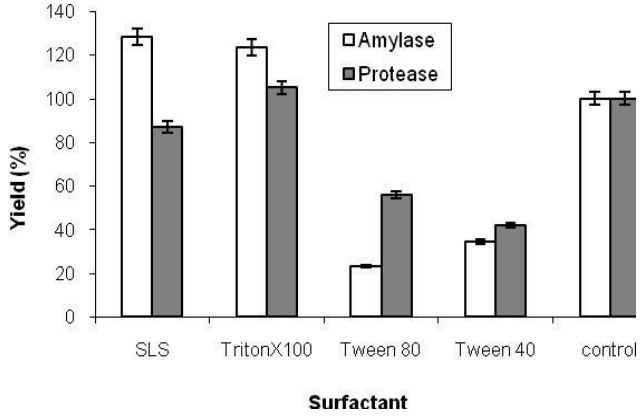

Figure 9. Effect of surfactants on the production of amylase and protease.

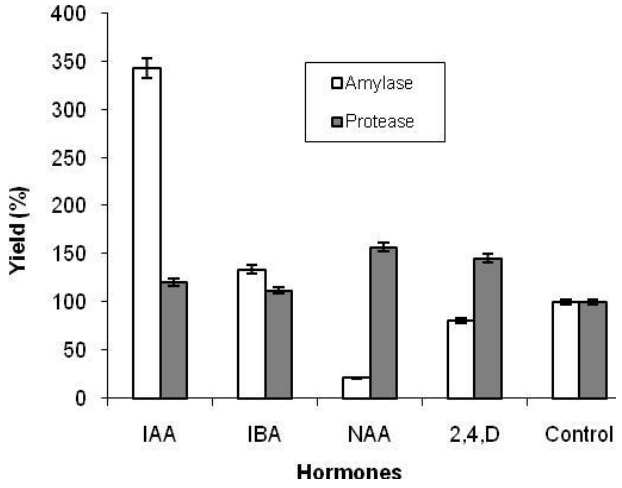

Figure 10. Effect of hormones on the production of amylase and protease.

acidic acid (NAA) and 2, 4, D enhanced protease production but reduced amylase production (Figure 10). IAA was more effective than IBA in enhancing production of amylase and protease.

Multienzymes production using engineered media

Culture was induced by cultivating it on different concentrations of casein and starch solution to get high yield. With starch alone, sporulation started within 8 to 10 days of incubation and high mycelia growth was also observed. Very little growth was observed with casein solution only. However, adequate sporulation was observed within 4 to 5 days with a mix of starch and casein (1:1 ratio) (Figure 11). With this engineered media amylase and protease yields had increased by 2.1 fold

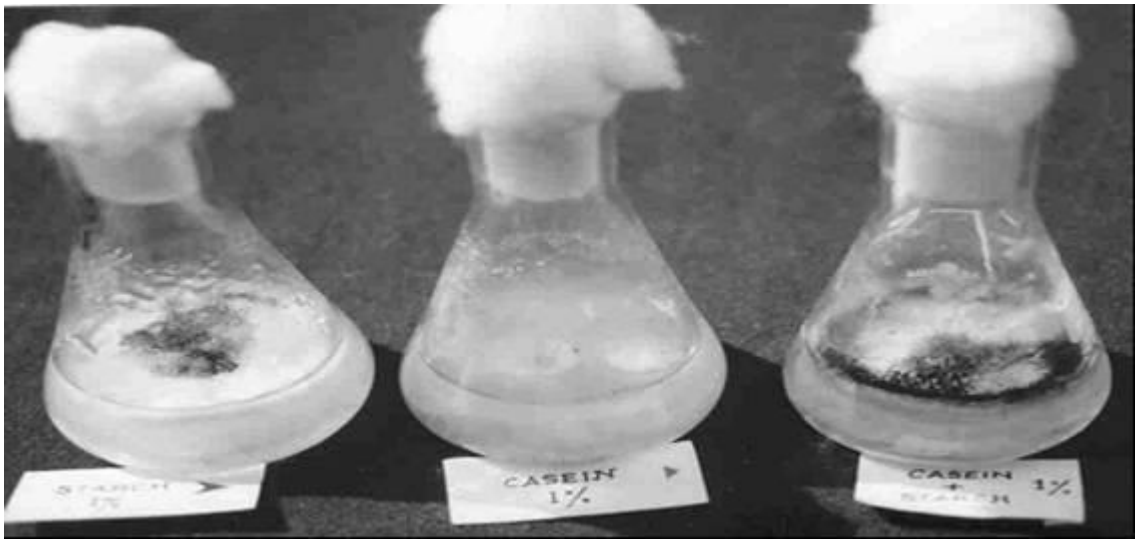

Figure 11. Effect of media engineering on the production of amylase and protease. (1% starch) (1% casein) (1% starch + 1% casein).

Table 3. Comparative analysis of yield of amylase and protease in different media.

			Yield (Fold)	
Media	**Amylase (U/gds)**	**Protease (U/gds)**	**Amylase**	**Protease**
Basal media (PM)	4528.4 ± 121	250.40 ± 10	1.0	1.0
PM + additives	8940.5 ± 198	829.0 ± 75	1.98	3.3
Engineered media (EM)	9420.6 ± 89	903.2 ± 55	2.1	3.61
EM + additives	9386.5 ± 101	934.8 ± 67	2.07	3.73

(9420.6 ± 89 U/gds) and 3.61 fold (903.2 ± 55U/gds), respectively, in comparison to basal media (Table 3).

Comparative study of yields of amylase and protease in different media

As shown in Table 3, basal media alone gave amylase and protease yield of 4528.4 ± 121U/gds and 250.4 ± 10 U/gds, respectively. Amylase and protease yields increased by 1.98 fold (8940.5 ± 198 U/gds) and 3.3 fold (829.0 ± 75 U/gds), respectively, with suitable additives in primary media. Yields of amylase and protease increased by 2.1 and 3.61 fold, respectively, with engineered media and by 2.07 and 3.73 fold, respectively, with additives in engineered media.

DISCUSSION

In present work *A. awamori* nakazawa was found as an effective producer of glucoamylases and proteases in solid state fermentation with wheat bran as substrate. *A.awamori* has been reported as an efficient producer of glucoamylase as well as acid proteases by Yamamoto et al. (2005). Aalbæk et al. (2002) reported production of multiple forms of glucoamylase (GA) and secretion of acid protease by *Aspergillus niger* in pH-controlled batch fermenters and chemostats. Al-Turki et al. (2008) reported improvement of glucoamylase production by *A.awamori* using mutagenesis. Ellaiah et al. (2002) and Yamamoto et al. (2005) have reported wheat bran as the best substrate among various agro-residues for production of glucoamylase by the *Aspergillus* sp. A3. Kaur et al. (2003) also reported production of α-amylase by *A. niger* using wheat bran in submerged and solid state fermentation. Production of glucoamylase by *A.awamori* in Solid-State Fermentation was also reported by Negi and Banerjee (2009).

In present study additional supplements to wheat bran, such as powder of peanut, corn, soybean seeds and sunflower seeds were not found necessary for enhancing concomitant production of amylase and protease because wheat bran with a composition: 15.5% protein; 64.51% carbohydrates; 4.2% fat and other nutrients also in desirable amount, probably provides most of the essential nutrients. Uyar and Baysal (2004) optimized production of alkaline protease employing *Bacillus* sp.

under solid state fermentation (SSF) and also reported highest enzyme production using wheat bran. Akpan and Adelaja (2004) reported significant increase in stability of amylase from *Aspergillus oryzae* using rice bran supplemented with soya bean flour (SBF) and cassava starch (3:1).

In solid state fermentation microbial growth and product formation occur at or near the surface of the solid substrate particle having low moisture contents, hence, it is crucial to provide optimum water content and control the water activity of the fermenting substrate Selvakumar and Pandey (1999). In present study (Figure 2), amylase and protease yields were optimum at substrate to Czapek-dox ratio of 1:1.5 (w/v), which indicates very little requirement of water for extracellular production of these enzymes.

Significant activities of both enzymes were noticed within 48 h of incubation while 96 h incubation had been found sufficient to get optimum yield of these enzymes. This is venerable and remarkable finding for enzymes industry, as not many species have been reported to perform with such efficiency in concomitant production of two enzymes at a time.

A.awamori was very efficient in wide range of temperature and pH. Optimum temperature and pH were 35°C and 5.5, respectively. Narayana and Vijaylakshmi (2008) has also reported optimum temperature and pH as 30°C and 6.5, respectively, for production of α-amylase from *Streptomyces albidoflavus*. Kaur et al. (2003) achieved optimum production of α-amylase during solid state fermentation (SSF) at pH 5.0 and 30°C. Yang and Lin (1998) have also reported maximum production of acid protease from *A. niger* at pH 4.0 and 30°C. Nahas and Waldemarin (2002) explored *Aspergillus ochraceus* for amylase production at pH 5.5 and 30°C.

Investigation on the impact of carbon and nitrogen supplements had revealed that not all carbon and nitrogen sources would act as enhancer for simultaneous production of these enzymes in a single fermentation system. Unlike in single enzyme production role of supplements become very critical in multi-enzyme production as not many supplements enhance simu-ltaneous production of all enzymes in a single bioreactor. Out of all the carbon sources investigated under this study, only lactose resulted in remarkable increase in yields of amylase as well as protease. Similarly, only peptone had been found as the most promising organic nitrogen source. Similar observations have also been made by Pandey et al. (2000), Cheng et al. (1995) in the case of protease and amylase production by different microbial species.

In many fermentation processes presence of metal ions may be inescapable and certain metal ions have been reported as enhancer of enzyme production. In present study only $HgCl_2$ was found enhancing the production of both the enzymes simultaneously. $CaCl_2$ appeared to act as a stabilizer for protease; which has also been reported earlier by Tunga et al. (2003), but greatly reduced activity of amylase. Among surfactants only Triton X-100 accelerated production of both enzymes, probably, because it enhances cell permeability and facilitates extra cellular enzyme secretion.

On comparative study of suitability of different media for concurrent production of amylase and protease in a single fermentation, engineered media with 1% casein and 1% starch was found as the most suitable media. Darani1 et al. (2008) also achieved optimum production of alkaline protease by *Bacillus* sp. 2 - 5 on date waste by addition of starch concentration of 5% (w/v or 0.5 g/L).

Conclusion

Concomitant production of amylase and protease by *A.awamori* in a single fermentation can be achieved by media engineered with 1% casein and 1% starch and maintaining various parameters at optimal levels. It is cost effective, convenient, less tedious and easier to scale up than the conventional method of using blend of enzymes from different fermentations. Production of amylase and protease in single fermentation can be particularly effective and useful for industries where both these enzymes are used together; such as food, detergent, pharmaceutical, oil extraction etc.

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