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Production of Amylases and Proteases by *Bacillus caldolyticus* from Food Industry Wastes

Thorsten Jamrath[1], *Christina Lindner*[1], *Milan K. Popović*[1]* *and Rakesh Bajpai*[2]

[1]Beuth Hochschule für Technik Berlin, University of Applied Sciences, FBV – Life Sciences and Technology, Seestrasse 64, DE-13347 Berlin, Germany

[2]University of Louisiana, Chemical Engineering Department, 131 Rex Street, 218 E Madison Hall, Lafayette, LA 70504, USA

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Summary

Amylases and proteases are utilized in industrial processes such as starch liquefaction or as supplements for washing agents. For these applications it is desirable to have enzymes active at high temperatures (>70 °C). In this work, thermostable α-amylase and neutral proteases were produced using the thermophilic strain *Bacillus caldolyticus* DSM 405. The goal of this work is to reduce the cost of production media by substituting expensive medium components such as prehydrolyzed starch and peptone, used in control fermentations, by inexpensive food industry wastes such as potato fruit water, potato pulp, cheese whey, draff, pea pulp, pea fruit water, bread residues, and pork blood. Comparative studies were conducted in shake flasks. With the use of such wastes, significant improvements in the activities of the enzyme α-amylase were obtained along with concomitant reductions in medium costs. With the use of pea pulp, 160 % increase in the activity of α-amylase was observed with 97 % reduction in medium costs compared to control medium. The cost of medium for the production of proteases also decreased by more than 50 %.

Key words: thermostable α-amylase, thermostable proteases, food industry wastes, *Bacillus caldolyticus*

Introduction

Industrial enzymes account for the second largest share of the market of fermentation products, behind the amino acids. In 2008, approx. 75 enzymes were traded with a sales volume of 15.9 billion US dollars (*1*). These enzymes are used in several different industrial sectors; nearly 50 % are utilized in the production of food and animal feed, followed by the detergent industry (with consumption of more than 30 %) and textile, paper, and fine chemical industries account for the rest (*2*). In biotechnological processes, hydrolases are the most commonly used enzymes. Of these, starch hydrolases (amylases) and protein hydrolases (proteases) represent the dominant enzymes. Forty percent of the enzymes produced are proteases (*3*) and 30 % are amylases (*4*). Several different amylases and proteases are known. The α-, β- and γ-amylases all act on the α-1,4 linkages in starches. These differ from each other in terms of their mode of action. The α-amylase (EC 3.2.1.1) acts randomly on any of the α-1,4 bonds in the starch chain, the β-amylase (EC 3.2. 1.2, predominant in plants) acts on the second α-1,4 bond from the end of the chain, and the γ-amylase (also known as glucoamylase or amyloglucosidase) acts on the first α-1,4 bond from the end of the starch chain. Another enzyme, α-1,6-glucosidase (an isoamylase), acts on the α-1,6 bonds in the starch chain. Of these, the most important industrial amylase is the α-amylase (*5*), which is used in food industry for liquefaction of starch during the production of high fructose corn syrup and for starch modification. It is used in washing powders, detergents,

*Corresponding author; Phone: ++49 30 325 7850; Fax: ++49 30 4504 3959; E-mail: popovic@beuth-hochschule.de

and in pulp and paper industry. α-Amylases from different sources exhibit different pH and temperature optima. Proteases are classified either as alkaline proteases or as neutral proteases depending on their pH optima. Alkaline proteases are used mainly in washing powders and detergents. Neutral proteases (EC 3.4.24.27/28) have broad applications in the food industry. They are used as meat tenderizer, for production of protein hydrolyzates or the sweetener phenylalanine, and other applications. Overviews of the biochemical properties of these enzymes have recently been published by several authors (*4–8*) and, therefore, are not presented here again.

Many industrial processes involve high temperatures. Hence, there is a need for thermostable enzymes. Such hydrolases can be obtained from thermophilic microorganisms, like *Bacillus stearothermophilus*, *Bacillus licheniformis* or *Bacillus caldolyticus* (*5–8*). Media used for the production of amylases generally involve complex carbon and nitrogen sources (*9*) that contribute substantially towards the costs of enzymes. This is not unusual as it has been reported that medium costs account for as much as 70 % of the raw material costs in extracellular enzyme fermentations (*10*). Therefore, the goal of this work is to evaluate industrial waste streams containing starch and organic nitrogen as substitutes for starch and soy flour/peptone in the fermentation media. Waste streams from several food industries have been selected for this purpose.

Some Food Industry Wastes in Germany

Starch-rich wastes

The food industry produces several wastes that are rich in carbohydrates and organic nitrogen in high amounts. A mass of 1.5 million tons of starch is produced annually in Germany (*11*). More than 60 % of this is obtained from potato, leaving potato pulp and potato fruit water as carbohydrate-rich waste streams. Annually, 275 000 tons of potato pulp (*12*) and 9 million tons of potato fruit water (*13*) are generated in Germany. At present, such streams are either used as animal feed/fertilizer or treated to reduce the biological oxygen demand (BOD) before discharge. Outside of the potato season, peas are used to produce starch and this production process results in generation of pea pulp and pea fruit water as wastes.

Other potential carbon sources include draff from the beer production and bread residue. German breweries produced 6.58 million m^3 of beer in 2009 (*14*) and generated 2 million tons of draff residue (oral communication with E. Hinzmann, German Brewers' Union, Berlin, Germany, 2011). The draff residue is presently used as animal feed. Bread residue is a common waste material resulting from the production of sliced bread (crusts and side pieces). Unsellable bread due to the approaching expiration date also contributes to bread residue. The latter amounts to 8–10 % (*15*) of the total bread production in Germany, which amounted to more than 4.5 million tons in 2009 (*14*). Furthermore, a lot of bread is thrown away in personal households as well – 2.6 billion slices of bread only in Great Britain (*16*). Bread residue is a good fermentation feedstock also since it is available year round and its availability is not associated with the harvest season.

Wastes rich in organic nitrogen

Corn steep liquor (residue from wet processing of corn), whey (waste stream from the production of cheese), and pork blood from abattoir refuses are potential nitrogen-rich wastes. Annual production of these wastes in Germany is over 16 million m^3 of whey (*17*), approx. 180 000 tons of dry corn steep liquor (*18*), and more than 180 million liters of pork blood (*19,20*).

The use of waste materials in fermentations has been reported by many researchers (*8,12,13,17,21–46*). Miscellaneous vegetable residues (*e.g.* apple pomace) have been transformed into flavors and fine chemicals by solid state fermentation, an overview of which has been presented by Laufenberg *et al.* (*21*). Similarly, ethanol has been produced from kitchen garbage (*22*), lactic acid from potato pulp (*13*), and microbial lipids from sweet potato (*47*).

Materials and Methods

Organism and media

Bacillus caldolyticus (DSM 405, DSMZ, Braunschweig, Germany) cells were pre-cultured in peptone-meat extract medium (5 and 3 g/L, respectively), supplemented with 1 g/L of $CaCl_2 \cdot 2H_2O$, in shake flasks in an orbital shaker at 70 °C for 8 h. Main cultures were conducted in 1-liter shaking flasks by adding 10 mL of preculture broth into 90 mL of culture medium and incubating again at 70 °C in an orbital shaker for 24 h. Nutrient medium containing in g/L: hydrolyzed starch 1, casein peptone 2, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 0.25, $FeSO_4 \cdot 7H_2O$ 30, $MnCl_2 \cdot 4H_2O$ 1.57, and $CaCl_2 \cdot 2H_2O$ 100 was used as control medium (*48*). The alternative media contained waste materials in place of hydrolyzed starch and/or casein peptone. The pH was adjusted to 7.0 in all the media at the start of fermentation.

Alternative media

The dosages of wastes in different media were based on the concentrations of starch and casein peptone in the control medium. Parts of the raw waste material were not soluble and, therefore, presumed not totally accessible to the microorganisms. Hence, the soluble starch content in the wastes was used to establish the concentration of wastes in alternate media for production of α-amylase. Similarly, soluble protein content in the wastes was the basis for determining the concentrations of waste in alternate media for the production of proteases.

Analysis of waste material

Waste materials were analyzed for total organic carbon (TOC) by catalytic combustion at high temperature followed by non-dispersive infrared detection (Rosemouth DC-190, Emerson Electric Company, Ferguson, MO, USA) of the carbon dioxide produced. Total bound nitrogen (TN_b) was measured using the test kit, Lanton LCK 338, Hach-Lange, Düsseldorf, Germany. Based on these results, the waste materials were categorized either as mainly carbon or as mainly nitrogen source.

Soluble starch and protein contents in the wastes were measured by incubating uninoculated solutions/suspensions of the wastes in control medium (without any starch and peptone) for 2 h at 70 °C followed by centrifugation at 12 000×*g* for 15 min. The concentration of dissolved proteins was measured in the supernatants using the method of Bradford (*49*). Soluble starch content in the supernatants was determined from the absorbance of starch-iodine complex at 620 nm using a calibration curve (*7*).

Fermentation and enzyme assays

All the fermentations were conducted in shake flasks either in duplicate or (mostly) triplicate. In all the cases, the waste materials were added to the media without sterilization. Incubations were conducted at 70 °C in orbital shakers at 150 rpm. During the experiments, no contaminations were observed by inspection under microscopes and it was suspected that the contaminations did not develop in spite of the use of unsterile waste materials due to the high fermentation temperature of 70 °C.

Several samples were analyzed from each shake flask. α-Amylase activity was measured photometrically, monitoring starch hydrolysation by culture supernatant stained with iodine, as introduced by Manning and Campbell (*50*) with modifications suggested by Bader *et al.* (*51*) and presented as units per mL (U/mL). Testing for other starch-hydrolyzing enzymes indicated total absence of glucoamylase, only traces of α-1,6 glucosidase, and no β-amylase. It is suspected that if any β-amylase was present, it was not thermostable (*6*).

Neutral protease activity was determined using the method of Strydom *et al.* (*52*) with substrate concentrations suggested by Iverson and Jørgensen (*53*), and reported as U/mL. One unit of protease activity is defined as the quantity of enzyme needed for degradation of 1 μmol of azocasein per minute.

Bacterial growth was monitored as cell count using an Abbe-Zeiss cell counting chamber (single square area 0.0025 mm^2; Brand GmbH, Wertheim, Germany).

Results and Discussion

Analyses of the different wastes and formulation of alternative media

Results of analyses of different wastes for total organic carbon (TOC), total bound nitrogen (TN_b), and soluble starch and soluble protein contents are presented in Table 1. All the waste materials contained carbon as well as nitrogen. Hence, each could be used as complete medium with the addition of minerals. Potato fruit water, potato pulp, draff, pea fruit water, pea pulp, and bread residue had high starch content and these were used as substrates for the production of α-amylase. The alternative fermentation media containing these starchy substrates are listed in Table 2 as media 1–7. Pork blood, corn steep liquor, and whey had high soluble protein content and were used for the production of proteases. These media are listed as alternative fermentation media 8–13 in Table 2. The control medium, with starch and peptone, is also listed in Table 2 for reference.

Table 1. Analyses of the waste materials

Substrate	TOC	TN_b	Soluble proteins	Soluble starch
	w/(mg/g)			
starch (hydrolyzed)	452	–	0	1000
peptone from casein	653	114	1000	0
potato fruit water	91	3.7	7.2	20.3
potato pulp	46	1.1	0.1	11.3
draff	172	8.6	0	17.9
pea fruit water	11	0.7	1.3	2.2
pea pulp	76	1.8	0.4	14.4
bread residue	263	2.4	0.13	51.7
	γ/(mg/mL)			
pork blood	306	28.2	52.5	0
corn steep liquor	245	31.1	19.3	0
whey	34	1.6	4.2	1.2

Table 2. Composition of wastes in the different fermentation media

Ferm. no.	Medium*	Dosage / 100 mL	*γ*(soluble starch) / g/L	*γ*(soluble proteins) / g/L
	control (starch+peptone)	0.1 g+0.2 g	1	2
1.	potato pulp	10 g	1.1	0.1
2.	potato fruit water	5 g	1	0.4
3.	draff	5 g	0.9	0
4.	pea pulp	10 g	1.4	0.3
5.	pea fruit water	10 g	0.2	0.1
6.	pea pulp+peptone	6.2 g+0.2 g	1	2
7.	bread residue	5 g	1.3	0.1
8.	pork blood	3.5 mL	0	1.8
9.	whey	50 mL	1.6	2
10.	corn steep liquor	10 mL	1	1.9
11.	pork blood (10 %)+starch	10 mL+0.1 g	1	5.3
12.	pork blood (5 %)+starch	5 mL+0.1 g	1	2.6
13.	pork blood (3 %)+starch	3 mL+0.1 g	1	1.6

*all the media contained mineral salts as per Brokamp *et al.* (*48*)

The amounts of wastes as starch alternatives in media 1–7 were estimated to provide roughly the same concentration of starch as in the control medium. In some cases, doing so resulted in soluble protein concentrations in the alternative media considerably lower than that in the control medium. As a result, medium 6 was designed with pea pulp supplemented with peptone to provide the same starch and peptone concentrations as in the control medium.

In media 8–13, the amounts of wastes were calculated to provide concentration of soluble protein approx. same as in control medium. Since pork blood had no soluble starch (medium 8, Table 2), different concentra-

tions of starch were also added to media 11–13 to provide the same starch concentrations as in control medium.

Relatively large amounts of the waste materials needed to be added to the different media (Table 2) since the raw waste materials were not in dried form and the contents of nitrogen and carbon in them were low compared to those in the dry components (starch and peptone) of the control medium.

Production of α-amylase from different carbon sources

Typical profiles of α-amylase activity in two different media (medium 1 and 7) are presented in Figs. 1 and 2, respectively. The enzyme activity in control medium is also presented in both figures. Error bars of triplicate experiments are also shown in these figures. Transient accumulation of α-amylase is evident and the enzyme activity decreased with continued incubation of cells with alternative media as well as with control media. Similar trends were observed for all the media (1–7) and in each case the activity of α-amylase in the fermentation broths peaked around 4–8 h from the start of fermentation. Potato pulp (medium 1) and pea pulp (medium 4) resulted in similar maximum enzyme activities that were around 2.6 times the values obtained in the control medium. The second highest peak value of α-amylase activity was observed in the bread residue medium (medium 7), which was about two times that in the control medium.

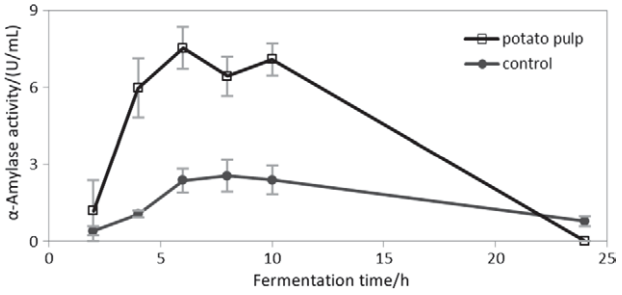

Fig. 1. Time course of extracellular α-amylase activity during the *B. caldolyticus* cultivation on potato pulp medium in comparison with control

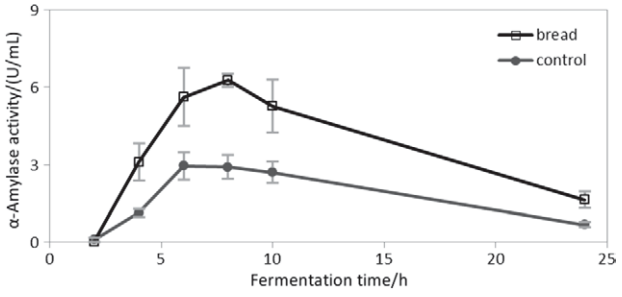

Fig. 2. Time course of extracellular α-amylase activity during the *B. caldolyticus* cultivation on bread residue medium in comparison with control

The maximum α-amylase activities, the cell counts and the corresponding time elapsed since the inoculation in different media (media 1–7 and control medium) are listed in Table 3. The data for the control medium are average values of 29 shake flask fermentations, whereas the others (media 1–7) are average values of three fermentations. It is clear that good cell growth could be achieved in alternate media 1–7 even when only one waste substance was used as main medium component replacing starch and casein peptone in the control medium. Furthermore, every waste C source supported very good α-amylase activities as well (Table 3); in all the cases, the enzyme activity was at least as good as in control medium. The best results were found with pea and potato pulp after 8 and 6 h of fermentation, respectively (up to 160 % increase over the control values). Bread residue medium resulted in 100 % increase in α-amylase activity over control medium after 6 h of fermentation. It is interesting that supplementation of pea pulp with peptone resulted in lower enzyme production than when only pea pulp was used. This confirms the observations of Schwab *et al*. (*7*) that optimal amounts of carbon as well as nitrogen sources are needed to obtain the best enzyme production by *B. caldolyticus*.

Table 3. Cell count, α-amylase activity and time elapsed since inoculation in fermentation media 1–7

Medium	Maximum α-amylase activity	Cell count	Time elapsed since inoculation
	U/mL	mL^{-1}	h
control (hydrolyzed starch and casein peptone medium)	3.3±1.8	$2.1\cdot10^9\pm2.9\cdot10^8$	9.8
1. potato pulp	8.4±0.7	$1.9\cdot10^9\pm2.6\cdot10^8$	6
2. potato fruit water	3.8±0.5	$1.3\cdot10^9\pm2.0\cdot10^8$	6
3. draff	3.1±1.4	$1.0\cdot10^9\pm1.5\cdot10^8$	6
4. pea pulp	8.6±1.8	$2.4\cdot10^9\pm5.4\cdot10^8$	8
5. pea fruit water	2.3±0.3	$1.4\cdot10^9\pm4.9\cdot10^8$	6
6. pea pulp+peptone	5.0±1.8	$1.3\cdot10^9\pm5.6\cdot10^8$	4
7. bread residue	6.7±0.7	$2.4\cdot10^9\pm6.5\cdot10^8$	6

Although we have not come across any publications relating to the production of enzymes by *Bacillus caldolyticus* using food industry wastes, efforts have been reported by ul-Haq *et al.* (*23*) for the use of low-cost agricultural products like wheat bran for the production of α-amylase. Wastes from the starch industry, like potato pulp, are even cheaper and these have been reported for the production of fine chemicals such as paramylon, lactic acid, and xylanase (*12*,*13*,*24*). Here we have shown that such wastes can be used as sole C and N sources for the production of α-amylase with *B. caldolyticus*.

Comparison of the α-amylase activities obtained in this work with those in other publications (*8*,*23*,*26*,*32*,*39*, *40*,*50*,*53*) is difficult, because different researchers have used different methods for determination of α-amylase activities and different methods result in vastly different assays of enzyme activity (*54*).

Production of protease from different nitrogen sources

As in the case of α-amylase activity, protease activities in different media too passed through a maximum

which was followed by reduction in activity with continued incubation. Even though cell counts in the various media were similar, the maximum protease activities in the alternative media were only a fraction of the activity produced in the control medium. As typical profiles, evolution of protease activity in the control medium and in medium 8 (an alternative medium containing 3 % pork blood) are presented in Fig. 3.

The observed maximum enzyme activities, the cell counts and the corresponding time elapsed since inoculation are summarized in Table 4 and compared with those

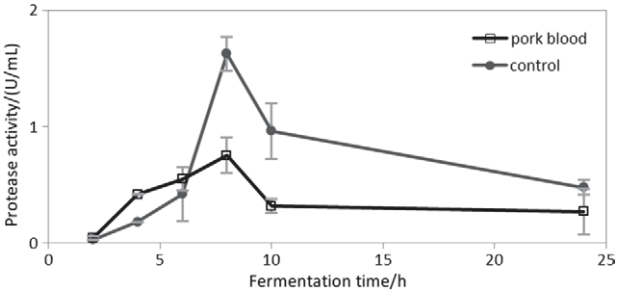

Fig. 3. Time course of extracellular protease activity during the *B. caldolyticus* cultivation on pork blood medium (3 %) in comparison with control

Table 4. Cell count, protease activity and and time elapsed since inoculation in the fermentation media 8–13

Medium	Maximum protease activity	Cell count	Time elapsed since inoculation
	U/mL	mL^{-1}	h
control (hydrolyzed starch and casein peptone medium)	1.2±0.5	$2.1\cdot10^9\pm6.3\cdot10^8$	8.8
8. pork blood	0.5±0.1	$3.6\cdot10^9\pm4.4\cdot10^8$	24
9. whey	0.6±0.1	$2.3\cdot10^9\pm9.5\cdot10^8$	6
10. corn steep liquor	0.5±0.1	$2.2\cdot10^9\pm3.5\cdot10^8$	6
11. pork blood (10 %)+starch	0.5±0.2	$3.6\cdot10^9\pm5.5\cdot10^8$	10
12. pork blood (5 %)+starch	0.5±0.3	$2.3\cdot10^9\pm6.5\cdot10^8$	10
13. pork blood (3 %)+starch	0.9±0.3	$1.4\cdot10^9\pm2.0\cdot10^8$	10

in control fermentations. Based on these results, it can be concluded that all the waste materials used as peptone replacement also permitted a good growth of *B. caldolyticus*. However, none of the substrates used in this work resulted in the increased protease production over that in control medium, as shown in Fig. 3 for medium 8 (3 % pork blood). The protease activity obtained with the wastes was at best 75 % of the average protease activity in control medium. The protease formation rate was partly delayed notably on the pork blood-based medium (medium 8), which leads to unfavorable productivity values. The delays were most significant when fresh pork blood was used as substrate. Obviously, there were certain inhibitory substances which delayed the growth of *B. caldolyticus* at 70 °C. Addition of hydrolyzed starch addressed this issue, but the negative effect on protease production persisted.

Dissolved proteins induce the production of proteases in many *Bacillus* strains (*55*) in the same way as dissolved starch induces the production of α-amylase. Proteases have successfully been produced with feather meal and corn steep liquor (*25*). Corn steep liquor is established for the cultivation of bacilli (*26*). As a result, besides vegetable waste substances, animal-based wastes such as pork blood are interesting fermentation substrates due to their high protein content and low price. But no positive effect was observed in this work.

Cost implications of the use of food wastes for production of enzymes

From the enzyme activities and the measured time of maximal activity, presented in Tables 3 and 4, productivity values for α-amylase and protease in the different media were calculated. The best were summarized in Table 5.

From our discussions with suppliers of the wastes, relative costs of starch in potato and pea pulp (with respect to the cost of pure starch) and of proteins in pork blood (with respect to the cost of peptone proteins) were available. These relative costs were used along with the medium compositions (Table 2) to determine the potential of unit cost savings with the use of alternative media containing these wastes. The costs of production media and other relevant numbers for these fermentations are presented in Table 5.

It is clear from Table 5 that the successful substitution of starch and peptone by starchy wastes reduces me-

Table 5. Comparison of medium costs and productivity in the production of enzymes

Enzyme	Medium	Relative volumetric medium costs	α-amylase activity	Relative costs for the same enzyme activity	Time of maximal activity	Enzyme productivity
		%	U/mL	%	h	U/(mL·h)
α-amylase	control	100	3.3	100	9.8	0.34
	potato pulp	2.58	8.4	1.01	8	1.05
	pea pulp	2.58	8.6	0.99	6	1.43
protease	control	100	1.2	100	8.8	0.136
	pork blood	15.3	0.5	36.7	24	0.02
	pork blood (3 %)+starch	46	0.9	61.5	10	0.09

dium costs (per volume unit) for α-amylase production by more than 95 %. At the same time, α-amylase activity also increased by 150 % over the value in control medium. As a result, the relative cost of medium on the basis of α-amylase activity could be reduced by 99 % by the use of potato or pea pulp. At the same time, the productivity of α-amylase increased fourfold.

For the production of proteases, the substitution of starch and peptone by proteinous wastes was not very successful as the maximum protease activity in the alternate media was at best only 75 % of that in the control medium. However, the wastes are significantly cheaper (cost of pork blood only medium was 15 % that of the control medium on volumetric basis). Even with the addition of hydrolyzed starch, the medium cost with pork blood (3 %) was only 46 % of the cost of control medium. Thus, there were attractive cost savings with the use of alternative media due to the low cost of pork blood in spite of the lower protease activities.

In a large number of industrial enzyme formulations, the enzymes are not purified beyond some concentration and precipitation. As a result, the use of industrial wastes as raw materials is not expected to change the costs of handling, packaging, and storage. Thus, one may expect the cost savings reported above to materialize. Additional cost savings may be achieved by considering a holistic concept of food production (27) by means of developing facility designs that incorporate segregating the waste streams at sources of production and upgrading those using processes such as fermentation.

Conclusions

Wastes from the food processing industry contain several reusable substances. Low prices for these materials coupled with high costs for classic substrates in cultivation media make a strong case for the use of wastes in fermentations. In this work, we demonstrated that several residues from the starch industry and other wastes can be successfully used for the production of α-amylase and proteases. Their use resulted in increasing productivity of α-amylase while decreasing feed costs substantially. Even when the productivity enhancement was absent as in case of proteases, the reductions in feed costs make use of wastes in fermentation media attractive.

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