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Optimization of Amylase Production from *Lactobacillus plantarum* CS by Submerged Fermentation Using Agro Wastes Substrates

George-Okafor Uzoamaka Ogechi, Nwachukwu Ujunwa Felicia*, Ezeme-Nwafor Amara

Department of Applied Microbiology and Brewing, Faculty of Applied Natural Sciences, Enugu State University of Science and Technology, Agbani, Nigeria

Email address:
nwachukwujunwa77@yahoo.com (N. U. Felicia)
*Corresponding author

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Abstract: Amylases are known to be of great importance worldwide. Most of their production using refined carbon and nitrogen substrates are expensive, resulting in the production of expensive goods which are not easily affordable by end users in our country Nigeria. This necessitates the call for the use of cost-effective substrates under optimized condition for maximum amylase yield. Hence, the present study was focused on the optimization of amylase production from *Lactobacillus plantarum* CS using agro wastes from local substrates. The parameters assayed were carbon and nitrogen substrates from agro wastes, fermentation period (24-168h), pH (3.5-11) and temperature (30°C-70°C). Others included solvents for preparation of fermentation medium and metal ions of Mg^{2+}, Mn^{2+}, K^{+}, Na^{+}, Hg^{2+}, Fe^{2+}, Cu^{2+} and Pb^{2+} as supplements. The amylase produced via shake-flask fermentation was assayed using Dinitro salicylic acid method. The result revealed 3% sweet potato and 1% bambara nut wastes as the best carbon and nitrogen components of the required fermentation medium of pH 6.5. The optimal amylase produced (38.6U/ml) was achieved within 48h at 35°C with 1% inoculum and Mn^{2+}/ Mg^{2+} as a co-factor. Relatively, Cu^{2+}, Pb^{2+} and Hg^{2+} reduced the amylase yield to < 68.13% as against Mn^{2+} and Mg^{2+} which enhanced the production to 121.66% and 101.09% respectively. Natural rain water used as solvent for the preparation of fermentation medium significantly enhanced amylase production (24.65 U/ml) in relation to other test waters at $P>0.05$. Comparatively, there was a significant increase in amylase produced under optimized condition than not. Conclusively, the obtained results which revealed high amylase yield with available and cost effective agro-wastes is promising for industrial application in Nigeria.

Keywords: Amylase, *L. plantarum* CS, Optimization, Agro Wastes, Fermentation

1. Introduction

Amylase is an enzyme that catalyzes the hydrolysis of any starchy molecules into sugars. It has been found to be among the most globally utilized enzyme especially in the starch processing industry [1, 2]. Other applications of amylases in food, detergent, pharmaceutical, textile and brewing industries have severally been reported [2-6]. Medical application of amylase also involves its utilization in pancreatic enzyme replacement therapy (PERT). It is one of the components that helps in the breakdown of saccharides into simple sugars [7]. Among the different types of enzymes known, amylase is one of the most vital enzymes in biotechnology and can be obtained from different sources including microorganisms [8].

Microorganisms are known to produce most of the industrial amylases especially α-amylases. The most widely reported microorganisms for the production of such amylases were actinomycetes, fungi and bacteria [2]. Actinomycetes such as *Nocardiopsis aegyptia* and *Streptomyces fragilis* DA7-7 have been associated with the production of thermostable amylases [2, 9]. Fungal amylases for commercial purposes include those from *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus oryzae* and *Aspergillus flavus* NSH9 that is thermally stable at 50°C [10, 11]. Bacteria capable of producing large amount of α-amylase

for industrial applications have been severally reported. Most importantly among them are thermostable amylases from *Bacillus* spp including *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* [12]. Some *Lactobacillus* spp including *L. plantarum* and *L. fermentum* have also been associated with amylase production [13]. The later organisms play very important roles in food industry as they are generally recognized as safe. So utilizing any of their strains in food industry including amylase production is safety assured. However, their production cost resulting from so many variables including expensive fermentation medium becomes worrisome.

Generally, most of the commercial production of microbial amylases require refined carbon and nitrogen substrates in their fermentation medium in mixed of other vital components. The organisms are provided with such adequate components and fermentation conditions for the expression of the desired product and the major goal of any industrial fermentation for any product is to maximize profit. To achieve this, optimization study from laboratory to industrial scale is very important. Recent studies on scale-up process have been carried out by optimizing various factors for high enzyme activity and yield [14, 15]. In addition to optimization process, the use of low-cost materials is another crucial factor for huge profit venture. These factors generated interest on the present study focused on the optimization of amylase production from *Lactobacillus plantarum* CS by submerged fermentation using agro-wastes from local substrates in Nigeria. The potential of the test agro-wastes in significant amylase production would subsequently boost Nigerian local industries including poultry feed processing at an economic rate.

2. Materials and Methods

2.1. Test Organism and Its Activation

The organism, *Lactobacillus plantarum* CS utilized in this study was previously isolated [16]. Its culture maintained on Nutrient agar slants at 4°C was activated in a fresh sterile DeMan Rogosa Sharpe (MRS) agar prior to use.

2.2. Preparation of Agro Wastes

The wastes tested in this study were those from yellow maize grain, yam, ripe banana and sweet potato peels as carbon sources and egg shell, defatted groundnut, bambara nut and soybean wastes as nitrogen sources. They were sorted, washed, dried under mild sun, homogenized using home blender (Corona), sieved with muslin cloth and stored in sterile containers prior to use.

2.3. Pre-optimization Fermentation for Amylase Production

Initial fermentation was carried out with freshly activated inoculum (1%$^{w}/_{v}$) in a constituted MRS fermented medium of pH 7 at 30°C for 72h [17]. The produced amylase was determined by Dinitrosalicylic acid (DNS) method as described by [18] but with modification in incubation period of 30min instead of 10min. The produced amylase was mixed with equal volume (1ml) of starch solution (pH 7) and incubated at 35°C for 30min. Thereafter, 2ml of DNS reagent was introduced into the enzyme mixture and boiled for 10min at room temperature. The final enzyme mixture made up to 10ml with distilled water was read at 540nm in a spectrophotometer. One unit (U) of produced amylase was defined as the amount of amylase that released 1mg of glucose from starch per minute under the applied condition.

2.4. Optimization Studies

The following parameters were studied:

2.4.1. Effect of Carbon and Nitrogen Sources from Agro-Wastes on Amylase Production

The test waste carbon substrates were assayed with glucose as control. The fermentation medium was constituted as described by DeMan Rogosa and Sharpe but with substitution of the glucose with 1% ($^{w}/_{v}$) of each test carbon substrate respectively. Thereafter, the 24h active grown *L. plantarum* cell (1% $^{v}/_{v}$) was introduced into each substituted fermentation medium. The shake-fermentation was first carried out at 30°C for 72h at 10x10000rpm as earlier described. The fermented broth was centrifuged at 10x 5000rpm for 15min and the cell free supernatant representing the crude enzyme was obtained. The amount of enzyme (U/ml) produced was confirmed through amylase activity assay as described above. Similar assay was also carried out for nitrogen test substrates that were respectively substituted for peptone in the fermentation medium. The best carbon and nitrogen waste substrates were further subjected to concentration assay for optimal enzyme yield.

2.4.2. Substrate Concentration Assay

The constituted MRS fermentation medium was prepared with varied concentrations (0.5-5%) of the best carbon that substituted glucose. The fermentation with 1% ($^{v}/_{v}$) of *L. plantarum* and amylase assays proceeded as earlier determined. Similar procedure was adopted for nitrogen concentrations (0.5-5%) which substituted peptone in the medium. The best carbon and nitrogen concentrations with optimal amylase activity were subsequently utilized for further optimization studies.

2.4.3. Temperature Profile on Amylase Production

The 1% ($^{v}/_{v}$) inoculum in a fermentation medium containing 3% of the best carbon and 1% of best nitrogen substrates were subjected to fermentation at different temperatures ranging from 20-70°C for 72h. The temperature that supported the maximum amylase activity was considered for subsequent studies.

2.4.4. pH Profile

The effect of pH on amylase production was studied by the use of fermentation media of varied pH of 3.5 to 11.5. The fermentation media of pH 3.5 to 7.5, and 8.5 to 11.5 were maintained with Citrate phosphate, phosphate and NaOH-phosphate buffers respectively. Fermentation was at optimal temperature of 35°C instead of 30°C for 72h. The fermented pH medium with maximum amylase activity was chosen for further assays.

2.4.5. Effect of Metal Ions on Amylase Production

The metal ions assayed were Mg^{2+}, Mn^{2+}, K^{+}, Na^{+}, Hg^{2+}, Fe^{2+}, Cu^{2+} and Pb^{2+}. Each of the metal ions (1% $^{w}/_{v}$) was incorporated into the determined fermentation medium prior to fermentation at 35°C for optimal period of 48h against initial 72h. Respective amylase activity was determined and the most effective metal was subsequently used.

2.4.6. Determination of Optimal Fermentation Period

The inoculum (1% $^{v}/_{v}$) in a fermentation medium of pH 6.5 containing 3% sweet potato and 1% bambara nut wastes among other constituents was subjected to shake-flask fermentation for 24 – 168h at 35°C. At every 24h, the amylase activity was assayed as earlier described.

2.4.7. Solvent Profile on Amylase Production

The fermentation medium was prepared using different types of water as solvent. The test waters were distilled, deionized and natural rain water. Thereafter, shake-flask fermentation with activated 1%($^{v}/_{v}$) *L. plantarum* culture was carried out at the determined optimal conditions. The enzyme activity was assayed as done earlier.

2.4.8. Inoculum Concentration Assay

The constituted MRS fermentation media of pH 6.5 containing 1% ($^{w}/_{v}$) of the best metal was prepared with the best solvent. They were respectively inoculated with 24h culture of *L. plantarum* at different concentrations ranging from 0.5 to 5.0%. Each shake-flask-fermentation was at 35°C for 48h. The amount of amylase produced was determined as earlier described.

2.5. Post-optimization Fermentation for Amylase Production

The obtained data after optimization assays were utilized for the final fermentation. The recovered crude amylase was measured as earlier described and compared statistically with the amylase yield prior to optimization.

2.6. Statistical Analysis

The student's t-test was performed for statistical analysis by using IBM SPSS Statistic 20 computer program. A P value of < 0.05 was considered to be statistically significant.

3. Results

All the obtained results were systematically presented in tables and figures.

3.1. Substrates and Their Concentration Profile on Amylase Production

The result revealed sweet potato and bambara nut wastes to be the best carbon and nitrogen sources among other test carbon and nitrogen wastes substrates for amylase production (figures 1 & 2).

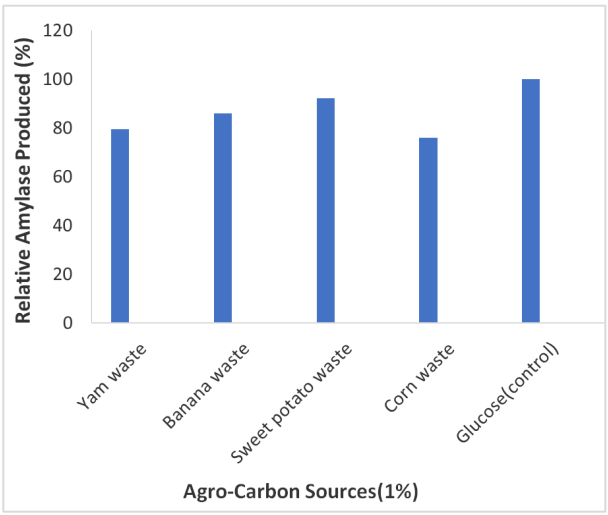

Figure 1. *Effect of organic carbon sources on amylase yield.*

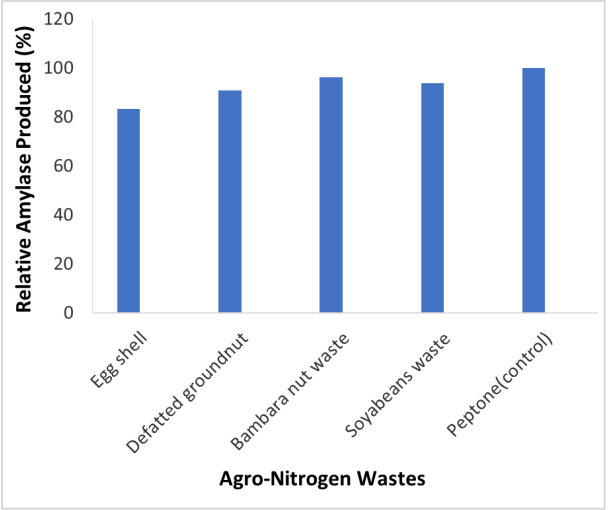

Figure 2. *Effect of organic nitrogen sources on amylase production.*

The substrate concentration profile of the fermentation medium indicated 3% sweet potato waste and 1% bambara waste as adequate for optimum amylase production by *L. plantarum* CS (figure 3).

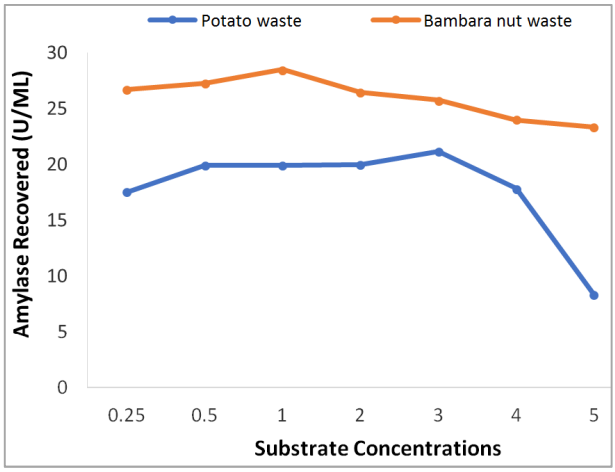

Figure 3. *Effect of substrates concentrations on amylase production.*

3.2. Temperature Profile on Amylase Synthesis

The result revealed maximum amylase production at 35°C (Table 1).

Table 1. *Effect of temperature on amylase production.*

Temperature (°C)	Amylase Recovered (U/ML)	*Relative Amylase produced (%)
30	15.92	73.19
35	21.75	100
40	15.98	73.47
45	15.50	71.26
50	15.07	69.27
55	14.12	64.92
60	13.55	62.29
65	13.40	61.61
70	10.88	50.02

*Relative enzyme production is expressed in comparison to the maximum enzyme produced which is taken as 100%.

3.3. Effect of pH on Production of Amylase

The optimum pH was observed at 6.5 (figure 4).

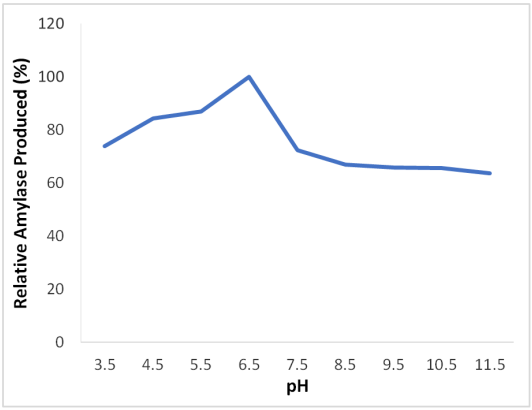

Figure 4. *Effect of pH on production of amylase.*

3.4. Fermentation Period in Relation to Optimal Amylase Production

The fermentation period for optimum production of amylase was revealed at 48h (figure 5).

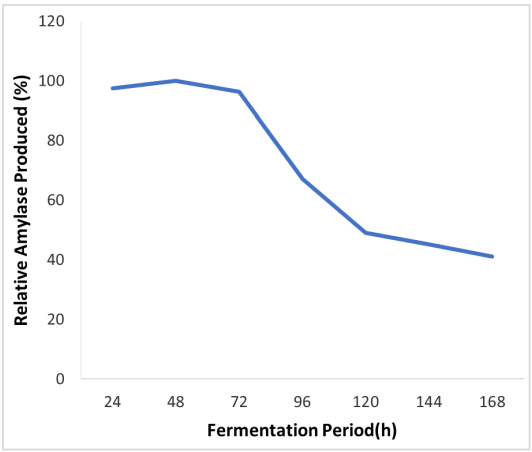

Figure 5. *Fermentation period in relation to optimal amylase production.*

3.5. Metal Ion on Amylase Yield

The optimal amylase produced was achieved with Mn^{2+} & Mg^{2+}. Relatively, Cu^{2+}, Pb^{2+} and Hg^{2+} reduced the amylase yield (Table 2).

Table 2. *Effect of metals on amylase production.*

Metals	Amylase Activity (u/ml)	*Relative Amylase produced (%)
Control	20.08	100
K^{+}	19.35	96.36
Pb^{2+}	10.87	54.13
Na^{+}	19.45	96.86
Mg^{2+}	20.30	101.09
Mn^{2+}	24.43	121.66
Fe^{2+}	20.03	99.75
Hg^{2+}	10.13	50.45
Cu^{2+}	13.68	68.13

*Relative amylase production is expressed in comparison with the yield from fermentation without metal ion (Control) and it is taken as 100%.

3.6. Inoculum Concentration on Amylase Production

Table 3 shows the effect of inoculum concentration (0.5 to 5%) on amylase production. Optimal amylase production was achieved with 1% (v/v) inoculum.

Table 3. *Effect of inoculum concentration on amylase production.*

Concentration (%)	Enzyme Recovered (U/ml)
0.5	27.82
1.0	30.03
1.5	26.43
2.0	20.73
2.5	14.15
3.0	13.83
3.5	13.60
4.0	10.47
4.5	10.40
5.0	10.03

3.7. Effect of Fermentation Water on Amylase Yield

The result revealed natural rain water to have the highest amount of amylase.

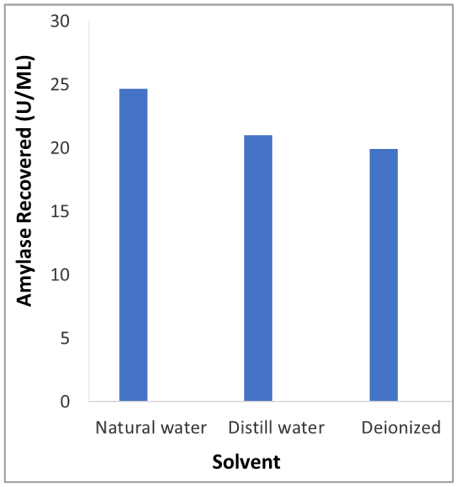

Figure 6. *Effect of fermentation water on amylase production.*

3.8. Post-optimization Production of Amylase

A significant higher amount of amylase (38.55U/ml) was recovered after optimization studies than during pre-optimized fermentation with amylase recovery of 22.50 U/ml ($p<0.05$) (figure 7).

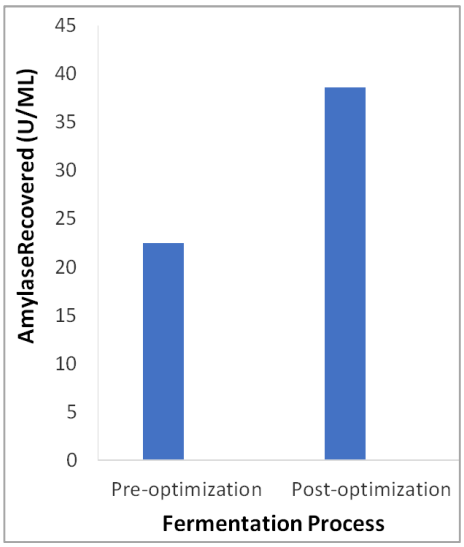

Figure 7. *Pre- and post-optimization production of amylase.*

4. Discussions

4.1. Substrates and Their Concentration Profile on Amylase Production

The relative amylase yields of the best carbon and nitrogen sources (92.08% and 96.26%) were lower than the yield (100%) obtained from the control fermentation with refined glucose and peptone as carbon and nitrogen sources (Figures 1 & 2). Interestingly, their differences were not significant ($p<0.05$). Considering the fact that there was no significant difference in amylase production from sweet potato and bambara wastes in relation to glucose and peptones, it then suggests there would be reduction in production cost if the costless waste substrates are utilized. This may consequently result to profit-making. The possible inducement of the amylase production by these substrates can promote their full hydrolysis when need arises. In addition, the possible use of these wastes as rich carbon and nitrogen sources would promote environmental waste management as it would provide avenue for the eradication of the accumulated wastes in the environment. The report by [19] on the environmental clean-up via the use of wastes for useful application supports the explanation. The use of agro-industrial wastes for amylase production by thermophilic actinomycetes and Coprinellus disseminatus had been reported [20, 21].

In the substrate concentration profile of the fermentation medium, 3% sweet potato waste and 1% bambara waste was adequate for optimum amylase production by *L. plantarum* CS (figure 3). This result is related to the reports of [22] that had highest amylase production using 2% potato peel. A higher needed carbon source concentration (3% sweet potato) than the nitrogen source (1% bambara waste) could be explained from the fact that carbon source is an energy supplier of the fermentation process. As most amylase enzymes are primary metabolites and growth dependent [23], the possibility of having high endothermic reaction and thus high energy utilization cannot be ruled out.

4.2. Temperature Profile on Amylase Synthesis

A maximum amylase production was observed at 35°C with a 50% enzyme loss at 70°C (Table 1). Similar results on maximum amylase production at 35°C were achieved by *Bacillus* spp and *Lactobacillus plantarum* MTCC [24, 25]. In contrast, optimal amylase was produced at 50°C by *Bacillus licheniformis* [26]. The ability of *L. plantarum* CS to produce maximum amylase at 35°C suggests the organisms to be a mesophile. Thus, the gradual temperature increase reduced the amount of amylase (Table 1), probably due to distorted systematic physiological activities leading to uncoordinated stress response by the mesophilic organism.

4.3. Effect of pH on Production of Amylase

The obtained result was similar to [27, 28] findings where maximum amounts of amylases were achieved in a fermentation medium of pH of 6.5. In contrary to this result, pH of 7 was reported to be the most effective for maximum amylase production by *L. plantarum* MTCC 1407 [25]. Further increase in pH beyond 6.5 led to a gradual decrease in amylase yield (Table 2). However, up to 73.8% and 63.6% relative amylase yields were recovered at pH 3.5 and 11.5 which are within acidic and alkaline range. This gave an indication that pH changes did not have tremendous effects on the amylase yield. This can be supported by [29] report which also indicated that fermentation pH which affected the growth of *L. plantarum* did not have much effect on its production of lactic acid. However, maximum amylase production at 6.5 by *L. plantarum* could be explained from the fact that the organism mostly preferred semi- neutral environment to others as [30] also reported optimum growth condition at 6.0.

4.4. Fermentation Period in Relation to Optimal Amylase Production

The time course study on fermentation for optimum amylase revealed 48h as the most effective period and this result is in line with reports from [31, 26] where highest amylase yields were also obtained within 48h. However, the findings of [32] had 72h fermentation period as best for *L. plantarum* amylase yield. The ability to obtain maximum amylase yield at 48h fermentation period indicated the producer to be a fast-grower with adequate potential for amylase production. It could also be due to optimized condition which facilitated both the growth of the organism and its metabolic synthesis. It is interesting to note that the *L. plantarum* was able to produce amylase with a relative activity of 97.4% within 24h. This positions *L. plantarum* as a good industrial organism.

4.5. Metal Ion on Amylase Yield

In enzyme production, metals demonstrate varied actions in their ability to act as effectors [33]. The presence of specific metal ion along with essential nutrient sources can inhibit or enhance amylase activity [34]. Therefore, the supplementation of the test metal ions into the production medium showed that Hg^{2+}, Cu^{2+} and Pb^{2+} inhibited amylase production (when compared to the activity of the control (fermentation without metal supplement) which is taken as 100% (Figure 4). The inhibition rate was higher in the presence of Pb^{2+} and Hg^{2+} ($\leq$ 54.13%) than that of Fe^{2+} and Cu^{2+} ($\leq$ 96.29%). Mn^{2+}significantly ($P>0.05$) enhanced amylase production (121.66%), followed by Mg^{2+} (101.09%) at optimal temperature of 35°C. This result can be compared with that from [35] which also implicated Mg^{2+} and Mn^{2+}as active co-factors of *Bacillus subtilis* for amylase production. The reduction of the amylase production by some of the test metals could be due to some thermal substrate denaturation at the assayed temperature. Contrarily, the Mg^{2+} and Mn^{2+} in the presence of sweet potato and bambara wastes must have offered considerable protection and stability to avoid heat denaturation. The enhancement of amylase yield by these two metals could be based on their ability to interact with negatively charged amino acids residues such as aspartic and glutamic acids [35, 36], probably present in the residues of the test nitrogen sources.

4.6. Inoculum Concentration on Amylase Production

The optimal amylase production was achieved with 1% (v/v) inoculum (table 3). As the inoculum concentration increased beyond 1%, there was a gradual reduction in amylase production. This study revealed that the concentration of inoculum plays a vital role in amylase production. The 1% concentration for optimal production is contrary to the findings of [37], which indicated maximum amylase production with 2% cell inoculum. A lower inoculum concentration for optimum amylase production observed in this study could be regarded as an economic advantage under industrial scale, as inoculum development would likely be less expensive.

4.7. Effect of Fermentation Water on Amylase Yield

Natural rain water yielded highest amount of amylase and this could be as a result of the presence of some minerals in it as natural water is known to contain some minerals including Mg ion. Lesser amylase yields from fermentation with distill and deionized water could then be attributed to the removal of some charged ions and minerals present in them since the produced amylase was stimulated by the presence some metals as determined in this study. Such enzymes require metal ions to maintain its stable and native state at high temperature [33].

4.8. Post-optimization Production of Amylase

The result shows that optimization studies carried out was effective since there was significant difference between the amylase produced during the pre- and post-optimized fermentation periods. This result supports the studies of [14, 15] which indicated that optimization of various fermentation factors leads to high enzyme activity and yield. Hence, the importance of optimization studies for industrial production.

5. Conclusion

The optimization study on the test parameters indicated a significant increase in amylase production at a low cost within 48h at 35°C in a fermentation medium (pH 6.5) containing effective agro wastes from sweet potatoes and bambara wastes as carbon and nitrogen sources against glucose and peptone which would be more expensive for industrial production. Supplementation with Mg^{2+} or Mn^{+} and the use of natural water as solvent also contributed to optimal amylase production. Hence, the obtained data are promising for future utilization especially in Nigeria after determination of other relevant parameters and scale-up processes.

6. Recommendation

Based on the results obtained from the study, agro wastes should not be discarded, rather, they should be utilized for large scale amylase production under above optimized conditions.

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