APPLE POMACE AS A SUBSTRATE FOR CELLULASE PRODUCTION BY *Bacillus macerans* UNDER SOLID STATE FERMENTATION

THESIS

by

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in

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Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan - 173 230 (H.P.) INDIA

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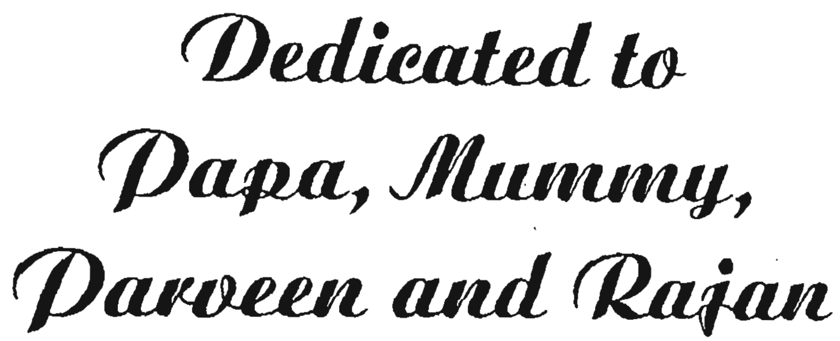

Dedicated to
Papa, Mummy,
Parveen and Rajan

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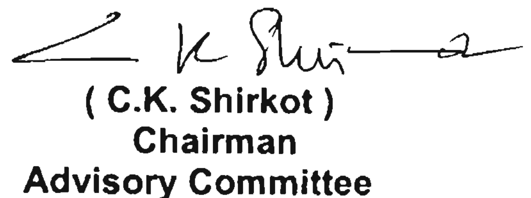
CERTIFICATE-I

This is to certify that the thesis entitled **"Apple pomace as a substrate for cellulase production by *Bacillus macerans* under solid state fermentation"**, submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **MICROBIOLOGY (BASIC SCIENCES)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.) is a bonafide research work carried out by **Ms. Shalu Sharma (F-99-19-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations have been fully acknowledged.



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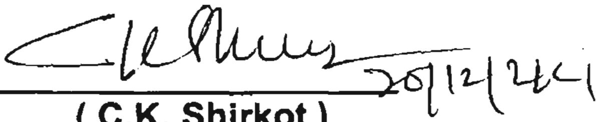
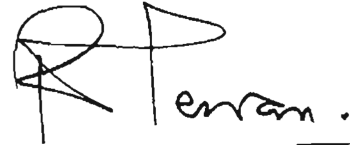
This is to certify that the thesis entitled **"Apple pomace as a substrate for cellulase production by *Bacillus macerans* under solid state fermentation"**, submitted by **Ms Shalu Sharma (F-99-19-M)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.), in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **MICROBIOLOGY (BASIC SCIENCES)** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.





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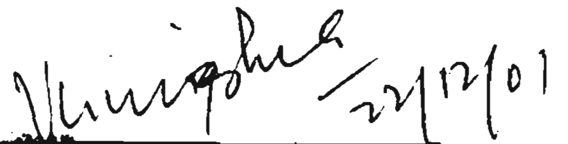

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Place : Nauni-Solan
Date : 24thNov. 2001

(*Shalu Sharma*)

CONTENTS

Chapter	Title	Page(s)
1.	Introduction	1-2
2.	Review of Literature	3-12
3.	Materials and Methods	13-21
4.	Experimental Results	23-54
5.	Discussion	56-64
6.	Summary and Conclusion	66-67
	References	(i)-(viii)
	Appendices	I-II

LIST OF TABLES

Table No.	Title	Page
1.	Evaluation of mineral salt medium for alkaline xylanase and cellulase production by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of inoculum	25
2.	Effect of particle size on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	26
3.	Effect of incubation period on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	27
4.	Effect of cooking temperature on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	28
5.	Effect of pH on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	31
6.	Effect of incubation temperature on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	32
7.	Effect of $(NH_4)_2$ SO_4 on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	33
8.	Effect of $NaN0_3$ on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	34
9.	Effect of yeast extract on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	37
10.	Effect of beef extract on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	38
11.	Effect of peptone on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	39

Table No.	*Title*	*Page*
12.	Effect of cellobiose on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	40
13.	Effect of carboxymethyl cellulose on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	41
14.	Effect of avicel on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	44
15.	Effect of KCl on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	45
16.	Effect of NaCl on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	46
17.	Effect of inoculum size on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation	47
18.	Combined effect of yeast extract, pH, inoculum size and carboxymethyl cellulose on extra cellular alkaline xylanase and cellulase production determined in 2^4 factorial experiment	48
19.	pH x CMC and pH x inoculum size – the two way table of xylanase and cellulase activity	51
20.	CMC x inoculum size and CMC x yeast extract – the two way table of xylanase and cellulase activity	52
21.	pH x yeast extract – the two way table of xylanase and cellulase activity	53
22.	Inoculum size x yeast extract – the two way table of xylanase and cellulase activity	53

Abbre 'iations Used

NH_4Cl	-	Ammonium chloride
$(NH_4)_2SO_4$	-	Ammonium sulphate
BSM	-	Basal Salts Medium
BSYEM	-	Basal Salts Yeast Extract Medium
β	-	Beta
$CaCl_2$	-	Calcium chloride
C	-	Centigrade
CMC	-	Carboxy methy cellulose
Cfu	-	Colony forming unit
$CuSO_4.5H_2O$	-	Copper sulphate
C.D.	-	Critical difference
DNSA	-	Dinitro sali cyclic acid
$FeSO_4$	-	Ferrous sulphate
KCl	-	Potassium chloride
$MgSO_4$	-	Magnessium sulphate
Na_2HPO_4	-	Di-sodium hydrogen phosphate
$NaNO_3$	-	Sodium nitrate
NaCl	-	Sodium chloride
g	-	Gram
h	-	Hour
l	-	Litre
µg	-	Microgram
µl	-	Microlitre
ml	-	Millilitre
mg	-	Milligram
M	-	Molar
N	-	Normal
OD	-	Optical density
%	-	Per cent
KH_2PO_4	-	Potassium dihydrogen phosphate
psi	-	Pounds $inch^{-2}$
rpm	-	rotation per minute
NaOH	-	Sodium hydroxide
HCl	-	Hydrogen chloride
S.E.	-	Standard Error
W/w	-	Weight by weight
W/v	-	Weight by volume
wt.	-	Weight
v/w	-	Volume by weight
nm	-	Nanometer

INTRODUCTION

Chapter-1

INTRODUCTION

Most of the agricultural and agro-industrial wastes contain three major polymeric components : cellulose, hemicellulose and lignin (Deschamps and Huet, 1985). Hemicellulose is one of the major components of lignocellulosic biomass and consists mainly of xylan (Chaudhary and Deobagkar, 1997). These are low molecular weight polysacharides that are found together with cellulose in plant tissues (He *et al.*, 1993) and both account for more than 50 per cent of plant biomass and therefore constitute the most abundant terrestrial organic molecules (Gilbert and Hazlewood, 1993). Xylan being major component of hemicellulose is present in both hard woods and annual plants and account for 20-35 per cent of the total dry weight in tropical biomass. In temperate soft woods, xylans are less abundant and may comprise about 8 per cent of the total dry weight (Srinivasan and Rele, 1999). Xylan is composed of a backbone of glycosidically β-1,4 linked xylopyranose units in all terrestrial plants.

Depending upon their source and mode of extraction, the xylan are substituted and may be linear or branched. Xylanase (E.C. 3.2.1.8) which is responsible for cleavage of β-1,4 linkage of xylan is produced by many microorganisms (Coughlan, 1992, Coughlan and Hazlewood, 1993).

In nature,microbial xylanases and associated enzyme degrade xylans present in lignocellulose. Potential applications of xylanases in biotechnology include wood biopulping, pulp bleaching by hydrolyzing xylan and rendering the lignin more exposed to bleaching chemicals, treating animal feed to increase its digestibility, processing fruit juices to increase their clarification and converting lignocellulosic substances into feed stocks and foods (Bajpai, 1997).

Cellulose present in the rapidly renewable ligno cellulosic waste is considered the most important reservoir of carbon for the production of glucose, fuel and chemical feed stock (Muniswaran and Charyulu, 1994). Cellulose is an unbranched glucose polymer composed of anhydrous D-glucose units with molecular weight ranging from 50,000 to 500,000 and each glucose molecule is linked by β-1,4 glucosidic linkage and these bonds can be hydrolyzed by cellulolytic enzymes.

Cellulases are a complex enzyme system comprising endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and β-D glucosidase. These enzymes, together with other related enzymes i.e. hemicellulases and pectinases are among the most important group of enzymes that are employed in the bioprocessing of cellulosic materials for feed, fuel, and chemical feed stocks. Cellulases also find application in the textile industry for fibre treatment and retting process (Pandey *et al.*, 1999).

The cost of the currently available cellulases, however, is too high for the economic viability of most of these processes. Development of an economical process for cellulase production is hindered mainly because of high cost of conventional substrates used for enzyme production as well as by the end product inhibition. These problems can be overcome, to some extent, by use of cheap substrate and employing the process of solid state fermentation as an alternative to submerged fermentation. Commercial cellulases are currently produced by submerged fermentation. However, use of solid state fermentation technique for the biosynthesis of cellulases and hemicellulases enzyme complex is gaining interest (Tengerdy, 1996; Archana and Satyanarayan, 1997; Voloktta, 2000). Solid state fermentation is a relatively simple, low technology operation, convenient and economical technique for the biosynthesis of cellulase enzyme complex. Therefore, the proposed studies are conducted with the objective to develop a suitable process for the production of cellulase(s) using apple pomace as solid substrate and employing *Bacillus macerans*.

REVIEW OF LITERATURE

Chapter-2

REVIEW OF LITERATURE

The information available on various aspects of present studies are reviewed below:

2.1 Apple pomace production and utilization

Apple pomace is a by-product of apple juice processing industry and at present constitutes a waste disposal problem. Apple processing industries generate as much as 25 per cent of original fresh fruit processed as pomace. Apple pomace is highly biodegradable and causes environmental pollution in addition to the loss of precious renewable resources. The approximate composition of apple pomace (Table 2.1) indicate that the apple pomace is a rich source of carbohydrate having 9.5 to 22.0 per cent of fermentable sugar. High fermentable sugar content makes it a good substrate for fermentation (Joshi, 1998).

The amount of initial sugar content depends upon the variety of apple processed, the processing conditions used and the amount of filter aids added (Hang *et al.*, 1982). Since it has high moisture content, it can be easily decomposed by microorganisms. Being rich in carbohydrates, fibres, acids and minerals its disposal as a waste in the environment is a huge loss of precious natural resources (Hang and Walter, 1989; Joshi and Joshi, 1990). However, in the past, efforts have been made to produce ethanol, citric acid, butanol and biogas from apple pomace and also to use it for cultivation of mushroom. There is a single report in literature where an attempt was made to use apple pomace as substrate for the production of extracellular xylanase and cellulase in liquid state fermentation (Bhalla and Joshi, 1993).

Table 2.1. Proximal chemical composition of apple pomace

Constituent	Wet weight basis	Dry weight basis
Moisture (%)	66.40-78.20	3.97-5.40
Acidity (%)	-	2.60
Total carbohydrates (%)	9.50-22.00	4.80-62.00
Glucose (%)	6.10	22.70
Fructose (%)	13.60	23.60
Sucrose (%)	-	1.80
Vitamin C (mg/100g)	-	18.50
Proteins (%)	1.0-1.8	4.45-5.67
Crude fibre (%)	4.3-10.5	4.70
Fat (ether extract %)	0.8-1.40	3.90
Pectins (%)	1.5-2.5	3.50
Minerals		
Potassium (%)	-	0.95
Calcium (%)	-	0.20
Sodium (%)	-	0.02
Magnesium (%)	-	0.02
Copper (mg/l)	-	1.02
Copper (mg/l)	-	1.10
Zinc (mg/l)	-	9.00
Iron (mg/l)	-	230.00
Calorific value	-	295.00

Source : Hang and Walter (1989); Garg *et al.*(1994); Rahmat *et al.*, (1995); Joshi and Sandhu (1996a)

2.2 Enzyme production on lignocellulosic substrates in solid state fermentation

Solid state fermentation (SSF) refer to the growth of microbes on solid substrates without the presence of free flowing water. All the biological activities ceases below the moisture content of 12 per cent and therefore, it is the minimum level at which solid state fermentation can proceed (Cannel and Young, 1980). Its main advantage is the sparing water in fermentation, in recovery and potential applications. The average overall cellulase production yield by SSF, as reported in literature (Tengerdy, 1996) ranged between 10-50 FPU g^{-1} DW substrate, which corresponds to 0.5-2.5 FPU ml^{-1} cellulase concentration in a 50 gl^{-1} slurry used in submerged fermentation. Lignocéllulosic material is obtained in large quantities as residual wastes in agriculture and forestry and constitutes an abundant but under utilized source of renewable biomass. A major part comes from barley, wheat, corn, rice, sugarcane bagasse etc. (Hartley *et al.*, 1987).

2.2.1 Cellulase production

Muniswaran and Charyulu (1994) carried out experiments for the production of cellulases by *Trichoderma viride* NCIM 1051 in solid state fermentation using coconut coir pith as a substrate. The highest FPase and carboxy methyl cellulase activities of 4.27 and 12.05 IUg^{-1}, respectively was obtained in 7 days of fermentation. An average substrate particle size of 375 μm resulted in better enzyme production.

Shamala *et al.* (1986) carried out experiments for the production of cellulases and xylanases using different fungal isolates i.e. *Aspergillus ustus*, *Sporotrichum pulverulentum*, *Trichoderma* sp. and *Botrytis* sp. in solid state fermentation using combination of wheat bran and rice straw. The maximum cellulase and D-Xylanase activity was with rice straw while wheat bran induced β-D-glucosidase activity.

Harchand and Singh (2001) reported the production of basal level of cellulase by *Streptomyces albaduncus* in the absence of cellulose. Highest yield of exoglucanase (0.473 IU ml^{-1}) and endoglucanase (6.40 IU ml^{-1}) was obtained on microcrystalline cellulose whereas β-glucosidase was induced maximally by delighified paddy straw (0.096 IU ml^{-1}). Paddy straw was found to be the excellent inducer of all the three enzymes studied.

The digestion of bacterial cellulose ribbons by mixture of enzymes consisting of cellulases viz. cellobiohydrolases Cel 6A, Cel 7A and endoglucanase Cel 45A from *Humicola insolens* was investigated. It was found that addition of minute quantities of endoglucanase Cel 5A resulted in a spectacular increase in saccharification whereas a moderate saccharification resulted from the mixture of Cel 45A and Cel 5A (Boisset *et al.*, 2001).

Nain *et al.* (2000) studied five strains of *Trichoderma viride* namely ITCC 1433, 1662, 2185, 3235 and 3277 in solid state fermentation of sorghum straw after adjusting C:N ratio to 35:1. It was found that all the strains utilized cellulose and carboxy methyl cellulose as carbon source and produced reducing sugar in the range of 0.38 to 1.60 mg sugar ml^{-1}. FPase activity was in the range of 0.04-0.09 units ml^{-1} with ITCC 1662 whereas CMCase activity ranged from 0.12 to 0.39 units ml^{-1}.

Dijkerman *et al.* (1997) studied the production of cellulolytic enzymes by *Neocallimastrix* sp., strain L2. Highest activities were found in filtrates from cultures grown on cellulose. By growing the fungus on inulin and lactose, it was found that it yielded the lowest cellulolytic activities. Highest specific activities for avicelase, endoglucanase, β-glucosidase and xylanase were obtained with avicel as a substrate.

Vares *et al.* (1995) used white rot fungus *Phlebia radiata* 79 (ATCC 64658) as inoculum for enzyme production of lignin peroxidase, manganese peroxidase and other lignolytic enzymes in solid state fermentation using wheat straw as substrate.

Sharma *et al.* (1995) reported cellulase production by *Penicillium chrysogenum* in solid state fermentation using wheat leaf and wheat stem as substrate. Wheat leaf was found to be superior than wheat stem for cellulase production.

The production of carboxymethyl cellulase, Xylanase and β-glucosidase by *Micrococcus* and *Cellulomonas* was carried out in solid state fermentation. Both the species grew well at 30°C and pH 7.0. It was found that *Micrococcus* sp. isolated from termite gut produced all the three enzymes (Saxena *et al.*, 1991).

Pardo and Forchiassin (1998) studied the effect of various nitrogen sources on production of cellulolytic enzyme system (endoglucanase, exoglucanase and cellobiase) of *Nectriacata*. Ammonium nitrate and ammonium tartarate resulted in the highest yield of cellulases. The optimal temperature of 24°C and initial pH of 6.5 gave highest yield of endoglucanase and cellobiase. However, optimal production of exoglucanase was obtained with initial pH 7.5.

Bacterial cellulase production by *Acetobacter xylinum* sub sp. *sucrofermentans* BPR 2001 was carried out in internal loop airlift reactor in air at an initial fructose concentration of 40 gl^{-1}. The bacterial cellulase production rate was 0.059 gl^{-1} per hour. When oxygen enriched air was supplied, the cellulase production rate increased to 0.093 gl^{-1} per hour and bacterial cellulase yield was enhanced from 11 to 18 per cent (Chao *et al.*, 2001).

Sun *et al.* (1997) developed a novel fed batch solid state fermentation process for cellulase production by *Trichoderma viride* using cellulosic substrate. The process could overcome the problems associated with high initial nutrient concentration while retaining advantages from the high total effective salt concentration.

Vipan *et al.* (1994) studied cellobiase production from a hyper cellobiase producing fungus *Schizophyllum commune*. Factors affecting the enzymatic saccharification were studied. A pH range of 4.0-5.0, temperature 45-50°C and substrate

concentration of 5.8-6.2 per cent were found to be optimum for enzymatic saccharification studies.

Romero *et al.* (1999) reported cellulase production by the fungus *Neurospora crassa* on wheat straw. The initial pH 6.5, incubation temperature 30°C and a straw concentration of 5 per cent yielded the higher activities of β-glucosidase, exoglucanase and endoglucanase. Two maxima of enzyme activities were observed for β-glucosidase and endoglucanase while exoglucanase activity was constant after exponential phase.

Voloktta *et al.* (2000) described the plate method for the detection of microorganisms with overall cellulolytic activity including the organisms like *Cytophaga*, in which the activity is cell bound. It was found that within a few days of incubation colonies of cellulose degrading bacteria formed holes in discs of lens paper placed on freshly incubated agar plates.

Fujian *et al.* (2001) studied the production of lignin peroxidase and manganese peroxidase in solid state fermentation using steam exploded wheat straw as substrate. It was found that the enzyme activities in solid state fermentation were higher than those obtained in submerged fermentation. Under optimal conditions of solid state fermentation, the maximum activities of the enzyme lignin peroxidase and manganese peroxidase were 2600 and 1375 UL^{-1}, respectively.

Gupta and Madamwar (1994) compared the production of cellulases and β-glucosidase on sugarcane bagasse, wheat bran and rice bran by *Aspergillus* sp. in solid state fermentation and submerged fermentation. The enzyme activities were higher in solid state fermentation and were further enhanced by alkali treatment of these cellulosic raw materials.

Bhalla and Joshi (1993) studied the production of cellulase and xylanase by *Aspergillus* spp. and *Trichoderma viride* on dried and pectin extracted apple pomace under solid and liquid state fermentation conditions. Maximum production of cellulase

Bhalla and Joshi (1993) studied the production of cellulase and xylanase by *Aspergillus* spp. and *Trichoderma viride* on dried and pectin extracted apple pomace under solid and liquid state fermentation conditions. Maximum production of cellulase (5.0 Units) and xylanase (4.2 Units) was obtained by *T. viride* and *A.niger*, respectively in dried apple pomace.

2.2.2 Xylanase production

Bacterial system are being increasingly investigated for the production of enzymes and metabolites by solid state fermentation. These fermentation system which are closer to the natural habitats of microbes may prove more efficient over the conventional methods in producing certain enzymes and metabolites. Reports on bacterial hydrolytic enzymes production by solid state fermentation, however, are primarily confined to *Bacillus* spp. which could be attributed to their ability to adhere to the substrate particles to produce filamentous cells for penetration and to their specific need for water activity (Archana and Satyanarayana, 1997).

Rajaram and Varma (1990) studied the xylanase production in solid state fermentation by *Bacillus thermoalkalophilus* using bagasse as a substrate. The enzyme preparation had activity optima at 60°C and 70°C and a half life of 60 minutes at 65°C. The enzyme was stable for 24 hours over a pH range of 4.0-6.0 while maximum activity was observed at pH 6.0-7.0. Enzyme production and activity were inhibited by the end product of xylan hydrolysis, xylose.

Subramaniyam and Prema (2000) used cellulose fibres as substrate and found that alkalophilic *Bacillus* SSP-34 produced more than 100 IU ml^{-1} of xylanase activity. The xylanase preparation had the optimum activity at temperature of 50°C and in a pH range of 6-8 with only small amount of cellulolytic activity i.e. CMCase 0.4 IU ml^{-1}, pH 7.0, FPase 0.2 IU ml^{-1}, pH 7.0 and no activity at pH 9.0.

Archana and Satyanarayana(1997) reported the production of extracellular thermostable cellulase free xylanase (E.C. 3.2.1.8) by *B.licheniformis* A-99 in solid state fermentation of wheat bran. The production of xylanase reached a peak in 72 hours at substrate moisture ratio 1:2:5 (w/v) and temperature 50°C.

Gessesse and Mamo (1999) found that *Bacillus* sp. AR-009 produced upto 720 Ug^{-1} of xylanase in solid state fermentation by using wheat bran as substrate. Xylanase production was highest at a wheat bran concentration of 10 g and moisture ratio of 1:0.5 to 1:1.5 and 10 per cent (w/w) Na_2Co_3 concentration.

Pereira *et al.* (2000) found that xylanase excreted by *Bacillus* sp. CCMI-966 exhibited high activity from pH 5.0-6.5 with the optimum at pH 6.0. The specific activity of purified xylanase was 137 U mg^{-1}.

Gawande and Kamat (1999) studied the production of xylanase with undetectable amount of cellulase by *Aspergillus terreus* and *A.niger* on various lignocellulosic substrate using solid state fermentation. Wheat bran was found to be the best substrate. Wheat bran moistened with mineral solution (1:5 w/v) containing 0.1 per cent yeast extract at incubation temperature of 35°C resulted in production of 74.5 IU ml^{-1} xylanase activity by *A.niger* and 68.9 IU ml^{-1} of xylanase activity by *A.terreus*.

Jain (1995) compared the production of xylanase and cellulase in solid state fermentation and submerged fermentation and it was found that there was higher xylanase yield in solid state fermentation when wheat bran and sugarcane bagasse were used as solid substrates by employing *Melanocarpus albomyces* IIS-68.

Dias *et al.* (1994) studied the xylanolytic activity of fungus *Dichomitus squaleus* by using pinus wood dust, newspaper strips and paper mill sludge as a source of carbon in an unshaken batch system.

Beg *et al.* (2000) reported the production of thermostable cellulase free xylanase from *Streptomyces* sp. in solid state fermentation using wheat bran and eucalyptus kraft pulp as prime solid substrates. The maximum xylanase yield obtained by using these two substrates were 2360 Ug^{-1} and 1200 Ug^{-1} at substrate moisture ratio of 1:3 and 1:2:5, respectively.

Xylanase production was found to be growth associated in 15 actinomycetes strains. Maximum activity was detected at the end of exponential phase and was stable throughout the stationery phase (Ball and McCarthy, 1989; Bachmann and McCarthy, 1991).

Thermoactinomyces thalophiles produced cellulase free extracellular endo-1, 4-β-xylanase at 50°C and pH 8.5. The crude enzyme had no traces of cellulase and maximum xylanase activity of 42 U ml^{-1} at 65°C and pH 8.5-9.0 was achieved using birch wood xylan as substrate (Kohli *et al.*, 2001).

2.3 Factors affecting cellulase(s) and xylanase production

Dekker (1983) found that much higher xylanase to endoglucanase activity ratio was obtained when *Trichoderma reesei* QN 123 was grown in the presence of substrate with high hemicellulose content (xylan). Cellulase production by species *Aspergillus ustus, Sporotrichum pulverulentum, Trichoderma* could be stepped up by adding ammonium sulphate (1%) to the substrate wheat bran and rice straw (Shamala *et al.*, 1985). Addition of sugars such as glucose, lactose, xylose, cellobiose during growth of *Clostridium sterocorarium* completely inhibited xylanase biosynthesis (Samain *et al.*, 1997).

The surfactants are known to increase the extracellular enzyme production in a number of organisms. In *Bacillus subtilis* enzyme production was enhanced by increasing concentration of surfactant (Tween 80) in the medium at a level of 0.1 per cent Tween 80, an increase of 6-fold in the enzyme yield was observed (Goes and

Sheppard, 1999). Yazdi *et al.* (1990) reported that the oleic acid and tween 80 increased the production of exoglucanases and endoglucanases by *Neurospora crassa* on micro-crystalline cellulose.

Lopez *et al.* (1998) reported that xylanase production by alkali-tolerant isolate of *Bacillus* was induced by xylose and xylan and was maximum at 42°C and pH 7.8.

MATERIALS AND METHODS

Chapter-3

MATERIALS AND METHODS

3.1 Basal salt medium

The basal salt medium (BSM) used had the following composition (per litre of distilled water) : 6.0g of Na_2HPO_4, 3.0g of KH_2PO_4, 0.5g of NaCl, 1.0g of NH_4Cl and Tween 20, 0.1% (v/w). Separately sterilized solution of 1M $MgSO_4$ (2ml) and 1 M $CaCl_2$ (0.1 ml) were added after the medium was autoclaved. BSM was supplemented with 0.5 per cent of yeast extract and this medium was designated as basal salt yeast extract medium (BSYEM). The medium was adjusted at pH 8.0 using 1 N NaOH or 1N HCl as the case may be.

3.2 Chemicals and Reagents

Analytical grade reagents obtained from BDH or E.merck were used for most of the investigations. Xylan (oat spelt),p-nitrophenyl-β-D-glucoside and Tween 20 were obtained from Aldrich Chemical Company, USA. Avicel was obtained from FMC Corporation, USA.

3.3 Microbiological Methods

3.3.1 Microbial strain and its maintenance

Culture of *Bacillus macerans* originally isolated from mushroom compost was obtained from the Culture Collection Centre of Microbiology Section, Department of Basic Sciences, Dr YS Parmar University of Horticulture & Forestry, Nauni, Solan (H.P). The bacterial culture was maintained in liquid as well as solid medium in BSYEM containing 0.5 per cent xylan and stored at 4°C.

3.3.2 Incubation

The culture was grown at 35°C except when mentioned otherwise.

3.3.3 Sterilization

Glassware used was thoroughly washed successively in detergent water, running tap water followed by rinsing in distilled water. The flasks used were tightly plugged with cotton gauge and covered with aluminium foils. Glassware was then kept in an oven at 180°C for 2 hours. All the media solutions unless mentioned otherwise, were sterilized at 15 psi pressure for 20 minutes. Heat labile chemicals like sugars were sterilized at 10 psi and were added separately to the medium. Laminar flow chamber was sterilized by ultraviolet irradiation for 15 minutes and occasionally swabed with formalin.

3.4 Viable count

Appropriate dilutions of bacterial population were used to seed the medium. The number of viable cells in the initial population were obtained by counting the number of colonies that developed after incubating the plates and multiplying by the dilution factor.

3.5 Substrates

3.5.1 Apple pomace

Apple pomace was procured from the Processing Unit of HPMC, Parwanoo, Himachal Pradesh. The oven dried material (60°C for 48 h) was ground in electric grinder and packed in air tight containers for subsequent studies. The powder was screened through ISI meshes and the fractions 10, 20 and 40 were collected.

3.6 Enzyme production

Basal salt yeast extract medium (BSYEM) with the composition given in 3.1 was used for the production of cellulase and xylanase by *Bacillus macerans*. Erlenmeyer

flasks (250 ml) containing 10g of substrate (apple pomace) and 20 ml of salt solution (BSYEM) were autoclaved at 15 psi pressure for 20 minutes, cooled and inoculated with 2 ml of bacterial suspension (O.D. 1.0 at 540 nm) and incubated at 35°C temperature in orbital incubator under unshaken condition. The flasks were gently tapped intermittently to mix the contents. At the desired intervals, the flasks were taken out and the contents were extracted with 45 ml sterilized buffer (0.2 M, pH 8.0, tris HCl). The flasks were kept in shaker for half an hour for proper mixing of apple pomace with buffer. The flask contents were centrifuged at 5300 rpm for 30 minutes at 4°C. The culture supernatant was used as crude enzyme preparation. Prior to centrifugation, the samples were withdrawn for determining viable number of cells by standard viable plate count technique.

3.6.1 Evaluation of mineral salt medium for cellulase and xylanase production

The production of cellulolytic and xylanolytic activity was studied after 72 h of incubation in 10 g of apple pomace supplemented with 20 ml Basal Salt Medium, Basal Salt Yeast Extract Medium and tap water. The fermentation medium that gave maximum enzyme yield was used in the further studies.

3.6.2 Effect of particle size on cellulase and xylanase production

Three different particle size of apple pomace viz. 10, 20 and 40 mesh size were used in order to find the particle size supporting the production of highest activity. The particle size that gave maximum activity was used for further studies.

3.6.3 Effect of incubation period on cellulase and xyulanase production

Effect of different incubation periods (0, 6, 18, 24, 48, 72, 96 h) was studied on the production of cellulolytic and xylanolytic enzymes. The incubation period that gave maximum enzyme production was selected for further studies.

3.6.4 Effect of cooking temperature of the substrate on cellulase and xylanase production

The cooking temperature used was 100, 110±1, 121±1 and 130±1°C and cooking time was kept 20 minutes. The temperature above 100°C were achieved by autoclaving the moist apple pomace at 5, 15 and 25 psi pressure for 20 minutes.

3.6.5 Effect of pH on cellulase and xylanase production

Initial pH of media was adjusted in the range of 4 to 10 using HCl/NaOH. All the flasks were incubated at 35°C. The pH that gave highest activity was used in further studies.

3.6.6 Effect of incubation temperature on cellulase and xylanase production

To find the optimum temperature for maximum xylanase and cellulase production, a range of temperature (20 to 60°C) was used to incubate the flasks. The temperature that gave highest cellulase and xylanase activity was used in further studies.

3.6.7 Effect of additional nitrogen sources on cellulaseand xylanase production

Various concentrations of ammonium sulphate and sodium nitrate (1, 2, 3 and 4%, mg w/w) were added to moist apple pomace for production studies. Also different concentrations of beef extract, peptone and yeast extract (0.25 to 2.5%, w/v) were added to apple pomace for enzyme production studies.

3.6.9 Effect of additional carbons compound on cellulase and xylanase production

The cellulase and xylanase production by *Bacillus macerans* in presence of different carbon compounds (1-4 mg %, w/v) cellobiose, Carbroxy methyl cellulose (CMC) and avicel was studied.

3.6.10 Effect of additional salts on cellulase(s) and xylanase production

Various concentrations (1 to 4 mg % w/v) of KCl, NaCl were added to moist apple pomace for enzyme production studies.

3.6.11 Effect of inoculum size on cellulase and xylanase production

5, 10, 20, 30 and 40 per cent (v/w) was added to the fermentation medium and the production of cellulase and xylanase activity was determined. The inoculum size showing the maximum activity was used for further studies.

3.7 2^4 Factorial experiment

For this purpose, instead of one parameter being varied, different combinations of optimum and next nearest level of each of these four parameters were used. The experiment was carried out with fixed substrate concentration of 10 g apple pomace and two values of carboxymethyl cellulose (2/3 mg %), pH (7.0/8.0), inoculum size (5 and 10%) and yeast extract (0.5/1.0%). Sixteen different culture conditions were attained by combination of four varying parameters. The experiment was performed in triplicates. The data obtained was subjected to statistical analysis. Treatment sum of square carrying 15 degree of freedom were further decomposed into 15 orthogonal contrasts (4 main effects; 6, two factor interaction; 4, three factor interaction and 1, four factor interaction) in order to have better insight into the behaviour of various factors affecting the xylanase and cellulase production.

3.8 Enzyme assay

The procedure used to estimate FPase, CMCase and xylanase activities essentially consisted of estimating reducing sugars formed by the action of respective enzymes on their substrates. The reducing sugar formed were estimated by calorimetric method of

Miller (1959) and the results are extrapolated by the standard curve drawn using glucose and/or xylose.

3.8.1 Filter paperase (FPase) Activity (Reese and Mandels, 1963)

Reagents:

i) Strips of filter paper (Whatman No.1)
ii) 0.2 M tris-HCl buffer pH 8.0
iii) Dinitrosalicylic acid (DNSA)
iv) Standard solution of glucose (1.0 mg/ml) in distilled water

Procedure

The reaction mixture contained 50 mg strip of filter paper (Whatman No.1) in 1 ml tris HCl buffer (0.2 M, pH 8.0) and 0.5 ml of diluted enzyme. The appropriate control for substrate and enzyme were also kept.

The mixture was incubated at 50°C for 1 h in water bath with occasional shaking. After incubation, 1 ml was drawn from the mixture and 3 ml of DNSA reagent was added into the test tube which also stopped the enzymatic reaction. The tubes were immersed in boiling water bath for 15 minutes. The tubes were removed and allowed to cool at room temperature. The contents were transferred to 25 ml volumetric flask and made 25 ml with distilled water. The optical density was read at 540 nm in Spectronic-20.

One unit of FPase activity was defined as the amount of enzyme that produced one micromole of glucose/ml/min, under the given assay conditions. FPase production is expressed as Ug^{-1} dry bacterial pomace (DBP). The enzyme assays were performed in triplicates with analytical grade reagents. The average values and standard errors were calculated.

3.8.2 **Carboxy methyl cellulase assay** (Reese and Mandels, 1963)

The reaction mixture contained 4.5 ml of 1.1 per cent CMC in tris HCl buffer (0.2 M, pH 8.0) of diluted enzyme. The mixture was incubated at 50°C for 1 hour. After the incubation, an aliquot (volume upto one ml) was withdrawn and added to 3 ml of DNSA which stopped the enzymic reaction. The tubes were immersed in boiling water bath for 15 minutes and then cooled at room temperature. The contents were transferred to 25 ml volumetric flasks and volume made up with distilled water. The optical density was read at 540 nm in a Spectronic-20.

One unit of enzyme activity was taken as that amount of enzyme which produced 4 mg of reducing sugars from CMC as the substrate in 1 h at 50°C. CMCase is expressed as Ug^{-1} dry bacterial pomace (DBP).

3.8.3 **Avicelase activity** (Berghem and Pettersson, 1973)

The reaction mixture contained 2.0 ml of 1 per cent suspension of Avicel in 0.2 M tris HCl buffer (pH 8.0), and 200 ul of enzyme solution. After incubation at 30°C for 2 hours, the mixture was filtered through Whatman No.1 filter paper and analyzed for reducing sugar by the method described earlier.

The unit of enzyme activity was defined as the amount of enzyme needed to liberate reducing sugar equivalent to 5 µg glucose under the assay conditions described above. Avicelase is expressed as Ug^{-1} dry bacterial pomace (DBP).

3.8.4 **β-glucosidase activity** (Berghem and Pettersson, 1973)

The substrate for the determination of β-glucosidase activity was P-nitrophenyl β-D-glucoside. The assay mixture contained 1.0 ml of 1 mM P-nitrophenyl β-D-glucoside in 0.2 M, tris HCl buffer buffer (pH 8.0) and 100 µl of enzyme solution. After incubation at 40°C for 10 minutes, 2.0 ml of 1 M Na_2CO_3 was added to the mixture to stop the

reaction. The mixture was diluted with 10 ml of distilled water and the P-nitrophenol liberated was determined from the absorbance at 400 nm. The standard curve was made from the stock solution of P-nitrophenol (80 μg/ml).

The unit of enzyme activity was defined as amount of enzyme which liberated 5 ug P-nitrophenol under the assay condition. β-glucosidase activity is expressed as Ug^{-1} dry bacterial pomace (DBP).

3.9 Xylanase assay (Dubey and Johri, 1987)

The reaction mixture contained 0.5 ml of 1 per cent D-xylan in tris HCl buffer (0.2 M, pH 8.0) and 0.5 ml of diluted enzyme. It was incubated at 50°C for 5 minutes in water bath with occasional shaking. After incubation 3 ml of DNSA reagent was added into the test tubes which also stopped the enzymatic reaction. The tubes were immersed in boiling water bath and removed after 15 minutes when colour development was completed. Tubes were cooled to room temperature. The contents were transferred to 25 ml volumetric flask and final volume was made with distilled water. Optical density was read at 540 nm in Spectronic-20.

One unit of enzyme activity was defined as the amount of enzyme that produced 1 micromole of reducing sugar per minute under given assay conditons. Xylanase activity is expressed as Ug^{-1} dry bacterial pomace (DBP).

3.9.1 Dinitrosalicylic acid (DNSA) sugar reagent (Miller, 1959)

Dinitrosalicyclic acid	-	10 g
Phenol	-	2 g
Na_2SO_4	-	0.5 g
NaOH	-	10 g
Sodium potassium tartarate	-	200 g

The reagent was prepared by dissolving solid components in required volume of sodium hydroxide and phenol with constant stirring. The solution was stored in coloured bottle in a refrigerator.

3.10 Statistical analysis

All the experiments were conducted in triplicates alongwith equal number of appropriate controls. The data obtained were subjected to analysis of variance technique using Completely Randomized Design developed by Gomez and Gomez (1976).

EXPERIMENTAL RESULTS

Chapter-4

EXPERIMENTAL RESULTS

The results obtained during the course of this study have been presented below:

4.1.1 Evaluation of mineral salt medium for alkaline xylanase and cellulase production

Bacillus macerans was grown on apple pomace supplemented with BSM, BSYEM and tap water as fermentation media. The results (Table 1) revealed that organism grew best on apple pomace supplemented with BSYEM followed by BSM and minimum growth was observed when fermentation media used was tap water. The maximum xylanase activity (500.1 Ug^{-1} DBP) and FPase (0.972 Ug^{-1} DBP) were obtained when BSYEM was used as fermentation medium.

4.1.2 Effect of particle size on alkaline xylanase and cellulase production

The effect of particle sizes on the production of extracellular cellulase and xylanase activity by *Bacillus macerans* is shown in Table 2. Maximum xylanase (535.6 Ug^{-1} DBP) and FPase (0.972 Ug^{-1} DBP) production was obtained when the particle size used was minimum (40 mesh size). The enzyme activities at all particle size was recorded to be significantly different. CMCase, avicelase and β-glucosidase were not detected.

The reducing sugar increased with decrease in particle size. The results revealed that the growth and enzyme activities concomitantly increased with decrease in particle size. The final pH of the medium decreased from pH 8.0 and ranged between 6.47 to 6.90.

4.1.3 Effect of incubation period on production of alkaline cellulolytic and xylanolytic enzymes

Growth of *B.macerans* and production of cellulolytic and xylanolytic activity was monitored for 72 h in 10 g of apple pomace (40 mesh size). It was observed that the FPase and xylanase activity was essentially present in the culture supernatant of the flask removed immediately after incubation i.e. 0 h (Table 3). The results indicated that the viable cells number and xylanase and cellulase production increased with increase in incubation period. The maximum concentration of viable cells (180 cfu x 10^5) was obtained at 72 h and coincided with the maximum xylanase (525 Ug^{-1} DBP) and FPase (0.972 Ug^{-1} DBP) activity. Further increase in incubation period decreased the growth as well as FPase and xylanase activity. CMCase, avicelase and β-glucosidase activities were not detected.

The concentration of reducing sugars in the culture supernatant increased during the fermentation with increase in incubation period and maximum amount (2171 μg $ml^{-1}g^{-1}$) was obtained at 72 h. The final pH of the medium decreased from initial pH 8.0 and ranged between 6.50 to 6.96.

4.1.4 Effect of cooking temperature on production of alkaline xylanase and cellulase

The experiment was conducted to determine the optimum cooking temperature for xylanase and cellulase production by varying the cooking temperature from 100±1 to 130±1°C. The results (Table 4) showed that the xylanase and FPase production was maximum at cooking temperature of 121±1°C. However, CMCase, avicelase and β-glucosidase activities were not detected. The production of xylanase and cellulase activity was found to be growth associated. The maximum xylanase (530.2 Ug^{-1} DBP) and FPase(0.972 Ug^{-1} DBP) activity was observed with maximum number of viable cells (200 cfu x 10^5).

Table 1. Evaluation of mineral salt medium for alkaline xylanase and cellulase production by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Moistening Agent	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
Tap water	370.2	0.387	N.D.	N.D.	N.D.	2000.0	143.7	6.65
BSM	400.0	0.583	N.D.	N.D.	N.D.	2001.0	183.7	6.58
BSYEM	500.1	0.972	N.D.	N.D.	N.D.	2171.0	196.0	6.61
$CD_{0.05}$	0.14	0.0009	N.D.	N.D.	N.D.	0.94	0.94	0.01
$S.E._m$	0.04	0.0002	N.D.	N.D.	N.D.	0.29	0.29	0.004

Reaction mixture

a) Xylanase : Substrate (1% D-xylan in tris HCl buffer 0.2 M, pH 8.0) = 0.5 ml; enzyme supernatant (diluted) = 0.5 ml; incubation temperature = 50°C; incubation period = 5 minutes

b) FPase : Substrate (50 mg strips of filter paper Whatman No.1 in tris HCl buffer 0.2 M, pH 8.0); enzyme supernatant (diluted) = 0.5 ml; incubation temperature – 50°C; incubation period = 1 hour

c) CMCase : Substrate (1.1% CMC in 0.05 M, pH 5.0 citrate buffer) = 4.5 ml; enzyme supernatant (diluted) = 0.5 ml; incubation temperature = 50°C; incubation period = 1 hour

d) Avicelase : Substrate (1% suspension of Avicel in 0.05 M sodium acetate buffer, pH 5.0) = 2.0 ml; enzyme supernantant = 200 μl; incubation temperature = 30°C; incubation period = 2 hours

e) β-glucosidase : Substrate (1 mM p-nitrophenyl-β-D-glucoside in in 0.05 M sodium acetate buffer, pH 5.0) = 1.0 ml; enzyme supernatant = 100 μl; incubation temperature = 40°C; incubation period = 10 minutes

Table 2. **Effect of particle size on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation**

Particle size (mesh size)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
10	199.7	0.194	N.D.	N.D.	N.D.	1401.0	138.0	6.90
20	311.6	0.387	N.D.	N.D.	N.D.	1751.0	174.0	6.83
40	535.6	0.972	N.D.	N.D.	N.D.	2171.0	181.0	6.47
$CD_{0.05}$ $S.E._m$	0.14 0.04	0.001 0.0002	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.48 0.42	1.99 0.57	0.01 0.003

Reaction mixture same as in Table 1

Table 3. Effect of incubation period on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Hours	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
0	102.5	N.D.	N.D.	N.D.	N.D.	750.3	13.6	6.96
6	225.7	N.D.	N.D.	N.D.	N.D.	875.3	43.0	6.94
18	235.0	0.194	N.D.	N.D.	N.D.	1156.0	90.3	6.74
24	300.7	0.194	N.D.	N.D.	N.D.	1401.0	110.7	6.60
48	375.2	0.389	N.D.	N.D.	N.D.	1716.0	140.7	6.50
72	525.2	0.972	N.D.	N.D.	N.D.	2171.0	180.0	6.43
96	224.5	0.387	N.D.	N.D.	N.D.	1645.0	160.3	6.40
$CD_{0.05}$ $S.E._m$	0.15 0.015	0.003 0.002	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.14 0.37	1.08 0.35	0.009 0.002

Reaction mixture same as in Table 1

Table 4. Effect of cooking temperature on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Cooking Temperature (°C)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
100	300.1	0.193	N.D.	N.D.	N.D.	1351.0	120.0	6.89
110 ± 1 (5psi)	374.1	0.388	N.D.	N.D.	N.D.	1716.0	168.3	6.70
121 ± 1 (15psi)	530.2	0.972	N.D.	N.D.	N.D.	2166.0	200.0	6.62
130 ± 1 (25psi)	224.1	N.D.	N.D.	N.D.	N.D.	1201.00	116.3	6.55
$CD_{0.05}$ $S.E._m$	0.13 0.03	0.001 0.0003	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.33 0.40	1.53 0.46	0.01 0.004

Reaction mixture same as in Table 1

The reducing sugar in the culture supernatant increased with the increase in cooking temperature and was maximum at 121$\pm$1°C. The final pH decreased with increase in cooking temperature and ranged between 6.55 to 6.89.

4.1.5 Effect of initial pH on production of alkaline xylanase and cellulase

The effect of varying initial pH (4.0 - 10.0) of the fermentation medium on the production of xylanase and cellulase activities was studied. It is evident from the results presented in Table 5 that all the fermentation parameters including xylanase and cellulase production were affected by initial pH of the medium. The fermentation parameter (except pH) increased drastically with increase in initial pH upto 8.0. On the other hand, the yield of xylanase and cellulase enzymes remained statistically at par at pH 6.0 and 9.0. The enzyme activity was significantly different from each other at all other pH values. The maximum viable cell number (199.7 cfu x 10^5), xylanase activity (535.1 Ug^{-1} DBP), FPase activity (0.971 Ug^{-1} DBP) and reducing sugars (2145 $\mu g\ ml^{-1}g^{-1}$) were observed at pH 8.0.

There was increase in the amount of reducing sugars in the culture supernatant with increase in pH and was maximum at pH 8.0. The final pH of the culture filterate was affected by the initial pH of the medium and ranged between 3.37 to 8.41.

4.1.6 Effect of incubation temperature on alkaline xylanase and cellulase production

A study on xylanase and cellulase production by *B.macerans* was conducted by incubating the flasks over a wide range of temperature at 20, 35, 40, 50 and 60°C using BSYEM with pH 8.0. As shown in (Table 6), increasing the fermentation temperature from 20°C to 50°C significantly affected the enzyme production, viable cell number, reducing sugar and final pH. The production of cellulase and xylanase activities decreased drastically at temperature values lower or higher than the optimum temperature of 35°C. Xylanase and cellulase activity was not detected at 60°C after 72 h of incubation. The production of xylanase and cellulase activity was found to be associated

with number of viable cells which increased upto 35°C and further increase in the temperature resulted in decrease in number of viable cells. The difference in the number of viable cells obtained at various temperatures was found to be statistically significant among themselves. CMCase, Avicelase and β-glucosidase activities were not detected.

The reducing sugar in culture filterate increased upto 35°C and decreased thereafter. The final pH of the culture supernatant ranged between 6.51 to 6.71.

4.1.7 Effect of $(NH_4)_2SO_4$ on alkaline xylanase and cellulase production

The effect of varying nitrogen concentration of $(NH_4)_2SO_4$ on xylanase and cellulase production was studied. The results presented in (Table 7) revealed that maximum xylanase (500 Ug^{-1} DBP) and FPase (0.776 Ug^{-1} DBP) activities were obtained at 3 mg per cent concentration of ammonium sulphate. A significant increase in the reducing sugar in the culture supernatant was observed on addition of ammonium sulphate over the control.

The reducing sugars in culture filterate increased with increase in ammonium sulphate concentration in the medium upto 3 per cent and decreased significantly at a concentration lower or higher than 3 per cent and the amount was at par at 4 per cent concentration. The final pH decreased and varied between 6.62 and 6.82.

4.1.8 Effect of $NaNO_3$ on production of alkaline xylanase and cellulase

The results on the effect of various concentrations (1-4 mg %) of $NaNO_3$ added to apple pomace on the production of extracellular xylanase and cellulase by *Bacillus macerans* is shown in (Table 8). Of the different concentrations used, 3.0 mg per cent $NaNO_3$ gave maximum xylanase (535.0 Ug^{-1} DBP) and cellulase (0.973 Ug^{-1} DBP) activity at 72 hrs. of incubation. Enzyme activity was found to be growth associated.

Table 5. Effect of pH on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

PH	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
4	235.7	N.D.	N.D.	N.D.	N.D.	841.0	100.0	3.37
5	245.1	0.193	N.D.	N.D.	N.D.	1261.0	140.7	4.13
6	254.6	0.387	N.D.	N.D.	N.D.	1470.0	16.0	5.14
7	375.1	0.583	N.D.	N.D.	N.D.	1731.0	189.0	6.52
8	535.1	0.971	N.D.	N.D.	N.D.	2145.0	199.7	6.73
9	254.7	N.D.	N.D.	N.D.	N.D.	1848.0	187.3	7.11
10	235.7	N.D.	N.D.	N.D.	N.D.	980.7	16.0	8.41
$CD_{0.05}$	0.19	0.001	N.D.	N.D.	N.D.	4.40	1.48	0.30
$S.E._m$	0.06	0.0003	N.D.	N.D.	N.D.	1.44	0.48	0.09

Reaction mixture same as in Table 1

Table 6. Effect of incubation temperature on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Incubation Temperature (°C)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
20	237.6	0.193	N.D.	N.D.	N.D.	1644.0	140.7	6.71
35	530.0	0.972	N.D.	N.D.	N.D.	2033.0	199.0	6.64
40	403.3	0.583	N.D.	N.D.	N.D.	1855.0	160.7	6.66
50	230.2	N.D.	N.D.	N.D.	N.D.	1261.0	100.3	6.56
60	N.D.	N.D.	N.D.	N.D.	N.D.	840.7	41.0	6.51
$CD_{0.05}$	0.57	0.001	N.D.	N.D.	N.D.	47.2	1.41	0.02
$S.E._m$	0.17	0.0002	N.D.	N.D.	N.D.	14.9	0.45	0.006

Reaction mixture same as in Table 1

Table 7. Effect of $(NH_4)_2SO_4$ on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

$(NH_4)_2SO_4$ (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	371.3	0.387	N.D.	N.D.	N.D.	1930.0	115.0	6.82
2	408.8	0.583	N.D.	N.D.	N.D.	2066.0	130.0	6.74
3	500.0	0.776	N.D.	N.D.	N.D.	2171.0	180.7	6.62
4	495.7	0.387	N.D.	N.D.	N.D.	2172.0	165.7	6.60
$CD_{0.05}$	0.15	0.001	N.D.	N.D.	N.D.	48.3	1.53	0.01
$S.E._m$	0.05	0.0002	N.D.	N.D.	N.D.	14.7	0.47	0.005

Reaction mixture same as in Table 1

Table 8. Effect of $NaNo_3$ on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

$NaNo_3$ (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	380.7	0.583	N.D.	N.D.	N.D.	1961.0	138.0	6.80
2	418.7	0.776	N.D.	N.D.	N.D.	2100.0	174.0	6.62
3	535.0	0.973	N.D.	N.D.	N.D.	2189.0	181.0	6.53
4	525.0	0.706	N.D.	N.D.	N.D.	2170.0	168.7	6.51
$CD_{0.05}$	0.17	0.001	N.D.	N.D.	N.D.	1.43	1.71	0.01
$S.E._m$	0.52	0.0003	N.D.	N.D.	N.D.	0.43	0.53	0.004

Reaction mixture same as in Table 1

The reducing sugar in the culture supernatant increased with increase in $NaNO_3$ concentration upto 3 per cent. The final pH varied between 6.60 to 6.82.

4.1.9 Effect of yeast extract on alkaline xylanase and cellulase production

An experiment was designed to determine the optimum concentration of yeast extract for maximum enzyme production. Different concentrations (0.25 to 2.5% w/v) of yeast extract were added to fermentation medium. The results (Table 9) revealed that production of xylanolytic and cellulolytic activities including other fermentation parameters were significantly affected by the addition of yeast extract. Increase in yeast extract concentration beyond 0.5% gradually decreased the production of xylanolytic and cellulolytic activities. The maximum reducing sugars (2175 $\mu g\ ml^{-1}g^{-1}$) xylanase activity (535.0 Ug^{-1} DBP), FPase activity (0.971 Ug^{-1} DBP) and number of viable cells (170 cfu x 10^5) were obtained in the presence of 0.5% yeast extract at 72 h of incubation.

The final pH of the culture filterate decreased and ranged between 6.49 to 6.69.

4.1.10 Effect of beef extract on alkaline xylanase and cellulase production

The varying concentrations of beef extract (0.25 to 2.5% w/v) were added to the fermentation medium and the results (Table 10) revealed that increase in beef extract concentration beyond 0.5% decreased the production of xylanase and cellulase activities. The maximum xylanolytic activity (475.00 Ug^{-1} DBP), FPase activity (0.706 Ug^{-1} DBP), reducing sugars (2134 $\mu g\ ml^{-1}g^{-1}$)) and number of viable cells (164 cfu x 10^5) were obtained at 0.5% beef extract concentration. CMCase, avicelase and β-glucosiase activities were not detected.

The reducing sugar in the culture supernatant decreased with increase in beef extract concentration. The final pH decreased and ranged between 6.53 to 6.63.

4.1.11 Effect of peptone on alkaline xylanase and cellulase production

An experiment was designed to determine the optimum concentration of peptone for maximum enzyme production. Different concentrations (0.25 to 2.5% w/v) of peptone were added in the fermentation medium. The results (Table 11) revealed that the production of xylanase and cellulase activities were affected by the addition of peptone. Increase in peptone concentration beyond 0.5% decreased the production of enzyme activities. The maximum xylanase (488.7 Ug^{-1} DBP) and FPase (0.387 Ug^{-1} DBP) activities were obtained at 72 h of incubation. However, the CMCase, Avicelase and β-glucosidase were not detected.

The reducing sugar in culture filterate decreased with increase in peptone concentration. The final pH ranged between 6.63 to 6.80.

4.1.12 Effect of cellobiose on alkaline xylanase and cellulase production

The results on the effect of different cellobiose concentrations (1 to 4 mg%) added to apple pomace on the production of extracellular xylanase and cellulase by *Bacillus macerans* is shown in Table 12. It was observed that addition of 3 per cent cellobiose gave maximum xylanase (500.1 Ug^{-1} DBP) and FPase (1.15 Ug^{-1} DBP) activities.

The reducing sugars in culture supernatant increased with the increase in cellobiose concentration. The final pH varied from 6.58 to 6.81.

4.1.13 Effect of carboxy methyl cellulose on production alkaline xylanase and cellulase

The effect of varying concentration of carboxymethyl cellulose (CMC) (1 to 4 mg %) on production of xylanolytic and cellulolytic activities was studied. The results revealed (Table 13) that the maximum xylanase (535.7 Ug^{-1} DBP) and FPase (1.35 Ug^{-1}

Table 9. Effect of yeast extract on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Yeast extract (% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
0.25	521.1	0.583	N.D.	N.D.	N.D.	2171.0	157.3	6.50
0.5	535.0	0.971	N.D.	N.D.	N.D.	2175.0	170.7	6.59
1.0	393.9	0.582	N.D.	N.D.	N.D.	2101.0	142.0	6.69
1.5	362.2	0.388	N.D.	N.D.	N.D.	1974.0	110.0	6.60
2.0	259.5	0.194	N.D.	N.D.	N.D.	1854.0	84.0	6.57
2.5	240.7	0.160	N.D.	N.D.	N.D.	1506.0	74.3	6.49
$CD_{0.05}$ $S.E._m$	0.19 0.06	0.001 0.0004	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.39 0.45	1.57 0.51	0.01 1.005

Reaction mixture same as in Table 1

Table 10. Effect of beef extract on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Beef extract (% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
0.25	474.9	0.197	N.D.	N.D.	N.D.	2133.00	157.3	6.51
0.5	475.0	0.706	N.D.	N.D.	N.D.	2134.0	164.0	6.53
1.0	380.2	0.582	N.D.	N.D.	N.D.	2064.0	135.3	6.62
1.5	362.0	0.274	N.D.	N.D.	N.D.	1854.0	109.3	6.70
2.0	259.3	0.196	N.D.	N.D.	N.D.	1575.0	81.0	6.71
2.5	240.7	0.190	N.D.	N.D.	N.D.	1294.0	70.6	6.63
$CD_{0.05}$	0.16	0.20	N.D.	N.D.	N.D.	1.67	1.11	0.01
$S.E._m$	0.05	0.06	N.D.	N.D.	N.D.	0.54	0.36	0.003

Reaction mixture same as in Table 1

Table 11. Effect of peptone on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Peptone (% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
0.25	485.1	0.197	N.D.	N.D.	N.D.	1999.0	139.7	6.59
0.5	488.7	0.387	N.D.	N.D.	N.D.	2100.0	149.7	6.63
1.0	393.8	0.293	N.D.	N.D.	N.D.	1994.0	129.0	6.67
1.5	362.0	0.197	N.D.	N.D.	N.D.	1855.0	97.3	6.71
2.0	259.3	0.190	N.D.	N.D.	N.D.	1644.0	74.3	6.75
2.5	222.0	N.D.	N.D.	N.D.	N.D.	1295.0	53.3	6.80
$CD_{0.05}$ $S.E._m$	0.19 0.06	0.0008 0.0002	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.57 0.51	1.57 0.51	0.01 0.003

Reaction mixture same as in Table 1

Table 12. Effect of cellobiose on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Cellobiose (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	369.5	0.387	N.D.	N.D.	N.D.	1574.0	143.3	6.81
2	412.4	0.584	N.D.	N.D.	N.D.	1750.0	160.7	6.76
3	500.1	1.150	N.D.	N.D.	N.D.	2241.0	170.3	6.58
4	485.1	0.972	N.D.	N.D.	N.D.	2240.0	167.3	6.75
$CD_{0.05}$ $S.E._m$	0.17 0.05	0.005 0.001	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.22 0.37	1.08 0.33	0.01 0.003

Reaction mixture same as in Table 1

Table 13. Effect of carboxymethyl cellulose (CMC) on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

CMC (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	384.4	0.387	N.D.	N.D.	N.D.	1715.0	142.0	6.83
2	454.0	1.160	N.D.	N.D.	N.D.	1785.0	168.0	6.70
3	535.7	1.350	N.D.	N.D.	N.D.	2275.0	186.0	6.60
4	530.1	1.30	N.D.	N.D.	N.D.	2273.0	169.0	6.56
$CD_{0.05}$ $S.E._m$	0.14 0.04	0.009 0.002	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.33 0.41	1.71 0.53	0.02 0.007

Reaction mixture same as in Table 1

DBP) activity was obtained at 3 mg % of CMC. Maximum number of viable cells (186 cfu x 10^5) were also observed with 3 mg % concentration of CMC.

Reducing sugar of culture filterate increased with the increase in concentration of CMC. The final pH of culture supernatant varied from 6.60 to 6.83.

4.1.14 Effect of avicel on alkaline xylanase and cellulase production

An experiment was conducted to determine the optimum concentration of avicel for maximum enzyme production. Different concentrations (1-4 mg%) of avicel was added to 10 g of apple pomace. The results (Table 14) revealed that 3 mg% of avicel gave the maximum xylanolytic activity (500.0 Ug^{-1} DBP) and FPase activity (1.157 Ug^{-1} DBP) as well as maximum cell number (174 cfu x 10^5). CMCase, Avicelase, β-glucosidase were not detected.

The reducing sugar increased with increase in the concentration of avicel. The pH varied between 6.61 to 6.86.

4.1.15 Effect of KCl on alkaline xylanase and cellulase production

Xylanolytic activity and cellulase activity was determined by adding various concentrations of KCl (1-3 mg%) into the fermentation medium. It was found (Table 15) that at 72 h of incubation, 3 mg% of KCl gave the maximum xylanase activity (494.7 Ug^{-1} DBP) and FPase activity (0.587 Ug^{-1} DBP) and the maximum viable number of cells observed were 184.3 cfu x 10^5.

The reducing sugars in culture supernatant was maximum at 3 mg% (2169.0 μg $ml^{-1}g^{-1}$) and the final pH varied between 6.60 to 6.86.

4.1.16 Effect of NaCl on alkaline xylanase and cellulase production

The effect of varying NaCl concentration (1-4 mg%) on the production of xylanolytic and cellulolytic activity revealed (Table 16) that the enzyme activity

increased with increase in NaCl concentration upto 3 per cent and thereafter activities declined. The maximum xylanase activity (505.9 Ug^{-1} DBP), FPase activity (0.971 Ug^{-1} DBP) and growth (188.3 x 10^5 cfu) was obtained at 3 mg per cent NaCl.

The amount of reducing sugars in culture filterate was maximum at 3 mg% NaCl (2171.0 µg $ml^{-1}g^{-1}$). The final pH of culture filterate varied between 6.58 to 6.74.

4.1.17 Effect of inóculum size on alkaline xylanase and cellulase production

The effect of varying bacterial concentration on the production of xylanolytic and cellulolytic activity was studied. The results (Table 17) revealed that when the inoculum size was increased from 5 to 10 per cent (v/w) the xylanase activity increased from (365.8 Ug^{-1} DBP) to (530.00 Ug^{-1} DBP) and the FPase activity increased from (0.193 Ug^{-1} DBP) to (0.971 Ug^{-1} DBP) at 72 h of incubation. CMCase, avicelase and β-glucosidase were not detected at any levels of the inoculum size used.

The initial inoculum size effected the final cell number obtained after 72 h of incubatiion as shown by statistically significant difference. The final cell number was significantly more when initial inoculum size used was 10 per cent.

The amount of reducing sugar increased with increase in inoculum size upto 20 per cent and thereafter decreased. The final pH varied between 6.60 to 6.68.

4.1.18 Combined effect of yeast extract, pH, inoculum size and carboxy methyl cellulose on extracellular alkaline xylanase and cellulase production determined in 2^4 factorial experiment

The results obtained from the factorial experiment performed with fixed substrate concentration of 10 g apple pomace per 20 ml of BSYEM are given in Table 18. Maximum xylanase production (1329 Ug^{-1} DBP) and FPase production (1.28 Ug^{-1} DBP) were obtained when the fermentation was carried at 0.5 per cent yeast extract, 10 per cent (v/w) inoculum size, 3 mg per cent (w/v) carboxymethyl cellulose and pH 8.0.

Table 14. Effect of avicel on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Avicel (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	369.7	0.194	N.D.	N.D.	N.D.	1576.0	135.3	6.86
2	425.1	0.973	N.D.	N.D.	N.D.	1750.0	165.7	6.74
3	500.0	1.157	N.D.	N.D.	N.D.	2170.0	174.3	6.61
4	480.5	0.972	N.D.	N.D.	N.D.	2169.0	168.3	6.57
$CD_{0.05}$ $S.E._m$	0.15 0.05	0.005 0.001	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.43 0.44	1.09 0.33	0.02 0.005

Reaction mixture same as in Table 1

Table 15. Effect of KCl on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

KCl (% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	352.7	0.193	N.D.	N.D.	N.D.	1611.00	140.3	6.86
2	408.7	0.387	N.D.	N.D.	N.D.	2060.0	159.0	6.74
3	494.7	0.587	N.D.	N.D.	N.D.	2169.0	184.3	6.60
4	480.4	0.386	N.D.	N.D.	N.D.	2168.0	168.3	6.58
$CD_{0.05}$	0.58	0.001	N.D.	N.D.	N.D.	1.21	1.33	0.02
$S.E._m$	0.18	0.0003	N.D.	N.D.	N.D.	0.37	0.41	0.003

Reaction mixture same as in Table 1

Table 16. Effect of NaCl on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

NaCl (mg% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
1	408.7	0.387	N.D.	N.D.	N.D.	2066.0	137.0	6.61
2	434.9	0.584	N.D.	N.D.	N.D.	2100.0	165.7	6.74
3	505.9	0.971	N.D.	N.D.	N.D.	2171.0	188.3	6.58
4	501.0	0.583	N.D.	N.D.	N.D.	2170.0	153.0	6.56
$CD_{0.05}$ $S.E._m$	0.15 0.04	0.0009 0.0002	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.21 0.37	1.33 0.41	0.01 0.002

Reaction mixture same as in Table 1

Table 17. Effect of inoculum size on production of alkaline xylanase and cellulase by *Bacillus macerans* during growth on apple pomace in solid state fermentation at 72 h of incubation

Inoculum size (% w/v)	Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Avicelase (Unit/ml)	β-glucosidase (Unit/ml)	Reducing sugar (μg/ml/g)	Viable count (cfu x 10^5)	Final pH
5	365.8	0.193	N.D.	N.D.	N.D.	1506.0	109.7	6.88
10	530.0	0.971	N.D.	N.D.	N.D.	1997.0	183.3	6.60
20	390.0	0.583	N.D.	N.D.	N.D.	2241.0	171.7	6.72
30	364.4	0.387	N.D.	N.D.	N.D.	1924.0	131.0	6.80
40	325.1	0.193	N.D.	N.D.	N.D.	1440.0	100.7	6.81
$CD_{0.05}$ $S.E._m$	0.12 0.03	0.001 0.0003	N.D. N.D.	N.D. N.D.	N.D. N.D.	1.55 0.50	1.48 0.47	0.01 0.003

Reaction mixture same as in Table 1

Table 18. Combined effect of yeast extract, pH, inoculum size and carboxymethyl cellulose on extra cellular alkaline xylanase and cellulase production determined in 2^4 factorial experiment

Factor combinations				Xylanase (Ug^{-1} DBP)	FPase (Ug^{-1} DBP)	CMCase (Unit/ml)	Aviceclase (Unit/ml)	β-glucosidase (Unit/ml)	Viable count (cfux 10^5)	Final pH	Relative activity Xylanase (%)	Relative activity FPase (%)
pH	CMC (%)	Inoculum Size (%)	Yeast Extract (%)									
7.0	2.0	5.0	0.5	867.7	1.150	N.D.	N.D.	N.D.	193.0	5.94	65.29	89.84
7.0	2.0	5.0	1.0	937.3	0.777	N.D.	N.D.	N.D.	195.0	5.93	70.53	60.70
7.0	2.0	10.0	0.5	980.3	1.160	N.D.	N.D.	N.D.	195.7	6.00	73.76	90.63
7.0	2.0	10.0	1.0	993.0	0.777	N.D.	N.D.	N.D.	197.7	5.97	74.72	60.70
7.0	3.0	5.0	0.5	993.1	0.776	N.D.	N.D.	N.D.	193.7	5.93	74.73	60.63
7.0	3.0	5.0	1.0	981.0	0.972	N.D.	N.D.	N.D.	194.0	5.90	73.81	75.94
7.0	3.0	10.0	0.5	993.1	1.150	N.D.	N.D.	N.D.	183.0	5.90	74.73	89.84
7.0	3.0	10.0	1.0	968.8	1.150	N.D.	N.D.	N.D.	186.3	5.93	72.90	89.84
8.0	2.0	5.0	0.5	1073.0	1.160	N.D.	N.D.	N.D.	191.3	5.83	80.74	90.63
8.0	2.0	5.0	1.0	1161.0	0.777	N.D.	N.D.	N.D.	192.0	5.53	87.36	60.70
8.0	2.0	10.0	0.5	1204.0	0.776	N.D.	N.D.	N.D.	191.0	5.43	90.59	60.63
8.0	2.0	10.0	1.0	1142.0	1.150	N.D.	N.D.	N.D.	197.0	5.31	85.93	89.84
8.0	3.0	5.0	0.5	1236.0	0.777	N.D.	N.D.	N.D.	194.3	5.32	93.00	60.70
8.0	3.0	5.0	1.0	1249.0	1.150	N.D.	N.D.	N.D.	196.0	5.30	93.98	89.84
8.0	3.0	10.0	0.5	1329.0	1.280	N.D.	N.D.	N.D.	201.0	5.37	100.00	100.00
8.0	3.0	10.0	1.0	1291.0	0.776	N.D.	N.D.	N.D	199.0	5.31	97.14	60.63

Reaction mixture same as in Table 1

* Relative activity is defined as 100% for the maximum xylanase activity of 1329 Ug^{-1} and FPase activity of 1.28 Ug-1 obtained by 3% CMC, 0.5% yeast extract, 10% inoculum size and pH 8.0.

The use of optimum level of yeast extract (0.5%), pH (8.0), inoculum size (10%) and carboxy methyl cellulose (3 mg %) made it possible to increase the xylanase activity value 2.5 times as compared to maximum activity obtained during the individual experiments. Similarly, the combined use of these parameters increased FPase activity from 0.972 Ug^{-1} DBP to 1.16 Ug^{-1} DBP.

The factorial experiment data was subjected to statistical analysis, the treatment comprising of all the combinations of optimum and immediate lower level of yeast extract, pH, CMC and inoculum size (16 combinations) were splited into four main factors : six, two factor : four three factor and one four factor interaction, each carrying single degree of freedom. How the production of xylanase and cellulase activity is affected by the levels of these factors and their combination is revealed by the analysis.

4.1.19 pH x CMC and pH x inoculum size - the two way table of xylanase and cellulase activity

The results obtained from two way table of pH x CMC and pH x inoculum size (Table 19) indicated that at both the levels of pH, the difference in average xylanase activity was statistically significant whereas FPase activity was non-significant.

The interaction studies showed that combination of high carboxy methyl cellulose with high pH value gave maximum xylanase activity (1276 Ug^{-1} DBP) and high CMC concentration with lower pH value gave maximum FPase activity (1.016 Ug^{-1} DBP). The interaction between pH x inoculum size revealed that combination of high pH value with high inoculum size produced maximum average xylanase activity (1242 Ug^{-1} DBP) and lower pH value with high inoculum size gave maximum FPase activity (1.063 Ug^{-1} DBP). The effect of pH and CMC on average FPase activity was non-significant whereas effect of inoculum size on FPase was found to be statistically significant.

4.1.20 CMC x inoculum size and CMC x yeast extract - two way table of xylanase and cellulase activity

The results obtained from two way table of CMC x inoculum size and CMC x yeast extract are presented in Table 20. The results showed that average xylanase activity obtained at inoculum size 10% (1113 Ug^{-1} DBP) was significantly more than that obtained at 5% inoculum size (1062 Ug^{-1} DBP). The average FPase activity was also more at 10% inoculum size (1.031 Ug^{-1} DBP) than that at 5% (0.945 Ug^{-1} DBP). Increase in CMC concentration from 2 to 3% increased average xylanase activity from (1045 Ug^{-1} DBP) to (1130 Ug^{-1} DBP) whereas FPase activity was increased from (0.985 Ug^{-1} DBP) to (0.991 Ug^{-1} DBP).

The effect of yeast extract on average xylanase and cellulase activity (Table 20) revealed that 0.5% yeast extract and 3% CMC gave maximum xylanase activity (1138 Ug^{-1} DBP) and 0.5% yeast extract and 2% CMC gave maximum FPase activity (1.065 Ug^{-1} DBP).

The effect of CMC, inoculum size and yeast extract was statistically significant on xylanse production. However, the effect of CMC on FPase was non-significant whereas the effect of inoculum size and yeast extract on FPase activity was statistically significant.

4.1.21 pH x yeast extract - two way table of xylanase and cellulase activity

The results from the two way table of pH x yeast extract are given in Table 21. The results revealed that 1.0% yeast extract and pH 8.0 gave maximum xylanase activity (1211 Ug^{-1} DBP) and 0.5% yeast extract and pH 7.0 gave maximum FPase activity (1.063 Ug^{-1} DBP). The average FPase activity was not affected by change in pH from 7.0 to 8.0 as revealed by the statistically non-significant difference in the activity.

Table 19. pH x CMC and pH x inoculum size – the two way table of xylanase and cellulase activity

CMC and Inoculum size	Xylanase PH 7.0	Xylanase PH 8.0	M. Mean	FPase pH 7.0	FPase pH 8.0	M. Mean
CMC 2%	944.6	1145.0	1045.0	0.969	1.001	0.985
3%	984.0	1276.0	1130.0	1.016	0.967	0.991
Marginal Mean	964.3	1211.0	Marginal Mean	0.992	0.984	
Inoculum Size 5%	944.8	1180.0	1062.0	0.921	0.969	0.945
10%	983.8	1242.0	1113.0	1.063	0.999	1.031
Marginal Mean	964.3	1211.0	Marginal Mean	0.992	0.984	

Effect	Xylanase S.E.m	Xylanase $CD_{0.05}$	FPase S.E.m	FPase $CD_{0.05}$
pH	0.08	0.23	0.08	0.24
CMC	0.08	0.23	0.08	0.24
Inoculum size	0.08	0.23	0.08	0.24
pH x CMC	0.11	0.26	0.12	0.28
pH x Inoculum size	0.11	0.26	0.12	0.28

Table 20. CMC x inoculum size and CMC x yeast extract – the two way table of xylanase and cellulase activity

Inoculum size and Yeast extract	Xylanase		M. Mean	FPase		M. Mean
	CMC			CMC		
	2%	3%		2%	3%	
Inoculum size 5%	1010.0	1115.0	1062.0	0.969	0.921	0.945
10%	1080.0	1145.0	1113.0	1.001	1.062	1.031
Marginal Mean	1045.0	1130.0	Marginal Mean	0.985	0.991	
Yeast extract 0.5%	1031.0	1138.0	1084.0	1.065	0.967	1.016
1.0%	1058.0	1122.0	1090.0	0.921	1.016	0.960
Marginal Mean	1045.0	1130.0	Marginal Mean	0.985	0.991	

Effect	Xylanase S.E.m	Xylanase $CD_{0.05}$	FPase S.E.m	FPase $CD_{0.05}$
CMC	0.08	0.23	0.08	0.24
Inoculum size	0.08	0.23	0.08	0.24
Yeast extract	0.08	0.23	0.08	0.24
CMC x Inoculum size	0.11	0.26	0.12	0.28
CMC x Yeast extract	0.11	0.26	0.12	0.28

Table 21. pH x yeast ex'ract – the two way table of xylanase and cellulase activity

Yeast Extract	Xylanase		M. Mean		FPase		M. Mean
	pH				pH		
	7.0	8.0			7.0	8.0	
0.5%	985.5	1210.0	1084.0		1.063	0.9680	1.016
1.0%	970.0	1211.0	1090.0		0.921	1.062	0.960
Marginal Mean	964.3	1211.0	Marginal Mean		0.992	0.984	

Effect	Xylanase S.E.m	Xylanase $CD_{0.05}$	FPase S.E.m	FPase $CD_{0.05}$
pH	0.08	0.23	0.08	0.24
Yeast extract	0.08	0.23	0.08	0.24
pH x Yeast extract	0.08	0.23	0.08	0.24

Table 22. Inoculum size x yeast extract – the two way table of xylanae and cellulase activity

Yeast Extract	Xylanase		M. Mean		FPase		M. Mean
	Inoculum size				Inoculum size		
	5%	10%			5%	10%	
0.5%	1042.0	1127.0	1084.0		0.968	1.063	1.016
1.0%	1082.0	1099.0	1090.0		0.921	0.999	0.960
Marginal Mean	1062.0	1113.0	Marginal Mean		0.945	1.013	

Effect	Xylanase S.E.m	Xylanase $CD_{0.05}$	FPase S.E.m	FPase $CD_{0.05}$
Inoculum size	0.08	0.23	0.08	0.24
Yeast extract	0.08	0.23	0.08	0.24
Inoculum size x Yeast extract	0.08	0.23	0.08	0.24

4.1.22 Inoculum size x yeast extract - two way table of xylanase and cellulase activity

The results obtained from two way (Table 22) inoculum size x yeast extract showed that 0.5% yeast extract and 10% inoculum size gave maximum xylanase activity (1127 Ug^{-1} DBP) and FPase activity (1.063 Ug^{-1} DBP). According to the results obtained, it was found that average xylanase and cellulase activity increased significantly with increase in inoculum size from 5 to 10%.

DISCUSSION

Chapter-5

DISCUSSION

Cellulases and associated plant cell wall degrading enzymes including hemicellulases and pectinases are increasingly employed in bioprocessing of plant material for fuel, feed and chemical feed stocks; in agriculture as silage additives, feed additives, retting additives and in the textile industry for fibre treatment. The cost of currently available commercial cellulases, however, is too high for economic viability for most of these processes (Tengerdy, 1996). To ensure the commercial utilization of cellulosic and hemicellulosic residues, the production of large quantities of cellulases and xylanases at low cost will be required. In this respect, the utilization of annually renewable agro-industrial wastes as a substrate represents an essential step to reduce the cost of enzyme production. Commercial cellulases are currently produced by submerged fermentation, most frequently employing genetically improved strains of microorganisms (Gayal and Kandeparkar, 1998).

The problem with cellulases and related enzyme production is the currently low yields, even with the best available mutant strains. The production cost may be lowered by using the process of solid state fermentation. The average overall cellulase production by solid state fermentation as reported in the literature (Tengerdy, 1996) is (10-50 $FPUg^{-1}$ dry weight) substrate as compared to the average production level of 3-20 FPU ml^{-1} in submerged fermentation. With optimization, strain selection and substrate selection, the productivity may be enhanced to higher level. The production of cellulases and xylanase in solid state fermentation by bacterial strains has been attempted by few workers (Archana and Satyanarayana, 1997; Pardo and Forchiassin, 1998). The ability to do so has been attributed to their characteristic to adhere to substrate particles, to produce filamentous cells for penetration and water activity requirements. In the light of numerous advantages of solid state fermentation, in this work, nutritional and cultural parameters have been optimized for the production of cellulases and xylanases by

alkalophilic strain of *Bacillus macerans* using apple pomace as substrate in solid state fermentation. The results obtained in the present investigations are discussed below.

Results obtained for the optimization of process parameters for solid state fermentation production of xylanolytic and cellulolytic enzyme with apple pomace as substrate demonstrated clearly the impact of process parameters on the yield of the enzymes as well as their independent nature in influencing the ability of *Bacillus macerans* to synthesize the enzyme.

As in the present study, basal salt yeast extract medium (BSYEM) was reported earlier to enhance the maximal xylanase (Sharma, 1998) production on apple pomace. A reduction in enzyme yield in basal salt medium (BSM) without yeast extract might be due to the reduction in growth of the organism as indicated by the presence of comparatively less number of viable cells.

The particle size (specific surface area) is a critical factor in solid state fermentation. Apple pomace particles of small mesh size favoured maximum enzyme production compared to larger particles (Table 2). This indicate that in solid state fermentation with small particle size sufficient surface area is available for adequate adsorption and filament penetration resulting in adequate sugar diffusion and have more bacterial growth and enzyme production. On the other hand, in the case of larger particles the reduced surface area/volume ratio provide a smaller surface for bacterial growth and might have inhibited absorption/penetration of bacterial cells on the substrate particles. These results are in agreement with those of Gupta and Madamwar (1994) who studied the effect of particle size on production cellulolytic enzymes by *Trichoderma viride* CCMI 9414. The results showed that maximum yield was obtained from finest particle size.

A similar trend was reported for glucoamylase with wheat bran (Pandey, 1990) and cellulase production with coir pith of small particle size (Muniswaran and Charyulu, 1994). With smaller particles the surface area for the growth is greater but the

interparticle porosity is less which is vice versa with larger particle size. These two opposing factors i.e. decrease in surface area and increase in porosity, probably interact to determine the values corresponding to the optimum growth and enzyme production (Krishna and Chandrasekhran, 1996).

Difference in the timing of appearance of xylanase and cellulase activity (Table 3) suggest that initial degradation of apple pomace is rapid. The increase in the production of xylanolytic activity with increase in incubation period from 48 to 72 h may be due to increase in the growth of the organism. This is supported by the direct relationship between the enzyme activity and final cell density. This observation is in agreement with the results obtained by Archana and Satyanarayana (1997) while working with *Bacillus licheniformis*.

In a previous study, the maximum xylanase and cellulase activities of *Bacillus* sp. AR-009 and *Penicillium funiculosum* were obtained after 72 h and 90 h, respectively, when viable cells were grown in wheat bran in solid state fermentation (Gessesse and Mamo, 1999; Gayal and Kandeparkar, 1998). Muniswaran and Charyulu (1994) reported maximum CMCase and FPase activities of 12.05 Ug^{-1} and 4.271 Ug^{-1}, respectively, obtained after 7 days of incubation by *Trichoderma viride* in solid state fermentation. There are some possible reasons for these differences including the strain of organism used, chemical composition of substrate, fermentation system and the conditions under which fermentation takes place.

The presence of xylanolytic activity at 0 h of incubation is an interesting finding. This could be due to the 1 h of incubation period during which the contents are allowed to mix thoroughly with the extraction buffer as mentioned in material and methods (3.6).

The presence of high amount of reducing sugars in fermentation medium seems to have not much repressing effect on xylanase and cellulase activity as the activity increases with the increase in amount of reducing sugars upto 72 h. The data indicate significant minimization of regulation of enzyme synthesis by end products in solid state

fermentation system. The ability of solid state fermentation to minimize catabolite repression has been documented (Ramesh and Lonsane, 1991; Dipti, 1994). The localized drop in substrate concentration in solid state fermentation system (Lonsane *et al.*, 1991) leads to enzyme production and concentration to its end products in the given regions in the medium. Such situation probably promotes preferential utilization of xylose formed and consequent drop in its concentration to non-regulatory levels. The slower and lower diffusion process in solid state fermentation system due to lower water activity, the adherence of bacterial cells to substrate particles might also have significant role to minimizing the regulatory mechanism (Ramesh and Lonsane, 1990) . Thus, the higher production of enzyme in solid state fermentation appear to be associated with lower level of enzyme end products. The decline in biomass concentration with increase in the incubation period from 48 h to 72 h may be due to reduced substrate availability, decrease in pH and accumulation of toxic products having inhibitory effect on bacterial cells.

As in the present study (Table 4), autoclaving at 121°C for 15 minutes was reported earlier to enhance maximal alpha-amylase production by *Bacillus subtilis* on banana waste (Krishna and Chandrasekran, 1996) and gibberelic acid production with wheat bran (Kumar and Lonsane, 1990). In the case of the solid state fermentation technique, autoclaving the moist solid medium cause some changes in the substrate and make it more amenable to degradation during fermentation. The yield of enzymes was lower with lower autoclaving temperature, indicate the insufficient modification of substrate for higher production of cellulolytic and xylanolytic enzymes. From the results, it is inferred that higher temperature of autoclaving above 121°C and pressure above 15 psi resulted in reduction in enzyme yield. Perhaps the substrate molecules which might have lost their structure of semicrystallanity when subjected to a higher temperature and pressure thereby decreasing the growth and enzyme yield.

The cellulase and xylanase production has been found to be highly dependent on pH and temperature. The maximum activity of FPase (0.971 Ug^{-1} DBP), xylanase (535.1 Ug^{-1} DBP), viable cell number (199.7 cfu x 10^5) and reducing sugar (2145 $\mu g\ ml^{-1}g^{-1}$)

46071

were obtained when *Bacillus macerans* was grown at pH 8.0. This shows the alkalophilic nature of the present bacterial strain producing enzyme active in alkaline range. Bansod *et al.* (1993); Fukumari *et al.* (1985) and Kroll (1980) studied the effect of pH on xylanase and cellulase production using wheat bran and micro-crystalline cellulose by *Cephalosporium, Bacillus* sp. and *Aeromonas*. It was found that found that maximum xylanase activity by *Cephalosporium* was obtained at pH 8.0 - 9.5; pH 8.0 supported maximum cellulase production by *Bacillus* spp. whereas *Aeromonas* produced maximum cellulase activity at pH 9.0. These differences were due to the strains of organisms used and nature of the substrate. The initial pH of the fermentation medium influenced the rate of enzyme production on banana fruit stock (Krishna and Chandrashekhran, 1996) and on wheat bran (Archana and Satyanarayana, 1997).

The present results clearly indicate that variation in pH of fermentation medium influenced the efficiency of the organism whatever the type of solid substrate used. The substantial decrease in final pH may be attributed to the production and accumulation of organic acids at elevated pH of the medium. Blakeman *et al.* (1988) reported that when the proportion of carbohydrates in fermentation medium is high e.g. as in fermentation of apple pomace, a pronounced drop in pH occurs. The decrease in pH of wheat straw due to bacterial fermentation have been reported to be due to release of hydrolytic enzymes and acid production in solid state fermentation process (Singh *et al.*, 1989; Kumar and Singh, 1990).

The optimum growth and production of cellulase and xylanase activity by *Bacillus macerans* occurred at 35°C (Table 6). The temperature optima for growth and xylanase production have been reported to mutually coincide at 60°C for *Bacillus stearothermophilus* (Khasin *et al.*, 1993); 37°C for *Bacillus circulans* (Esteban *et al.*, 1982) and 50°C for *Bacillus licheniformis* (Archana and Satyanarayana, 1998). The optimal temperature recorded for maximal enzyme production by *Bacillus macerans* in solid state fermentation is identical to that reported for *Cellulomonas* and *Micrococcus* sp. growing on carboxymethyl cellulose, bagasse and cellobiose (Saxena *et al.*, 1991).

These results indicate the independent nature of the temperature effect irrespective of the type of substrate used. The usual temperature maintained in solid state fermentation system is in the range of 25-32°C depending on the growth kinetics of the microorganism employed for the fermentation purposes (Lonsane *et al.*, 1991).

Highest xylanase titres in bacterial system have earlier been reported to occur generally at temperature that are optimum for the growth of organism (Sudgen *et al.*, 1994; Biswas *et al*:, 1990). However, maximum alpha-amylase production by *Bacillus megaterium* 16 M occurred at 35°C, although optimum temperature for growth was 40°C (Ramesh and Lonsane, 1987a).

The ability of the organism to produce an appreciable higher level of xylanase activity in presence of nitrogen supplements (Tables 7 to 11) indicate that apple pomace do not contain enough nutrients as source of nitrogen necessary for optimum growth and enzyme production by *Bacillus macerans*. Apple pomace has been reported to be poor source of protein (Garg *et al.*, 1994; Joshi and Sandhu, 1996a).

Addition of organic and inorganic sources of nitrogen enhanced the enzyme production significantly has also been observed in the earlier studies (Krishna and Chandrasekran, 1997; Pandey *et al.*, 1995). The present results advocate nitrogen enrichment of apple pomace fermentation medium for maximal xylanase production.

Addition of organic source of nitrogen in the form of yeast extract (Table 9) supported comparatively more growth alongwith maximum production of FPase (0.971 Ug^{-1} DBP) and xylanase (535 Ug^{-1} DBP). These results are in agreement with those obtained by Chandra *et al.* (1980); Babu and Satyanarayana (1993). The addition of yeast extract, beef extract and peptone had stimulatory effect on enzyme production presumably due to the presence of certain growth promoting nutrients and cofactors. The addition of yeast extract and polypeptone has been reported to stimulate growth of *Bacillus* sp. C-11 (Ikura and Horikoshi, 1977; Ganju *et al.*, 1990).

It is interesting to note that the ability of *Bacillus macerans* to produce FPase and xylanase activity was enhanced in the presence of 3% additional cellobiose in the fermentation medium (Table 12). The data indicate significant minimisation of regulation of enzyme synthesis by the end products of cellulases in solid state fermentation system. The strong inhibition of cellulase production by cellobiose in liquid state fermentation by *Streptomyces albaduncus* seems to be due to catabolite repression (Harchand and Singh, 2001). The ability of solid state fermentation to minimize catabolic repression has been documented (Ramesh and Lonsane, 1991; Dipti, 1994).

It is interesting to note that production of xylanase and FPase is enhanced by addition of carboxy methyl cellulose and avicel with corresponding increase in the growth as well as reducing sugars in the fermentation medium (Tables 13 & 14). Several explanations have been suggested to account for an increase in xylanase production when organisms are grown on cellulosic materials. It is possible that some cellulases acts in a non-specific manner and cleave the β-1,4 linkage of xylan molecule resulting in apparently higher xylanase activity. It has been demonstrated that many microorganism produce xylanases when cultured on cellulose (Dekker and Richard, 1976; Mishra *et al.*, 1987 and Saxena *et al.*, 1991). This may be due to cellulase having multi-substrate activity.Cellulases have been reported as multi adaptive enzymes and multiplicity of such enzymes may imply that pseudoxylanase activity is produced when organisms are grown on pure cellulose. The induction of cellulases by xylan containing cellulosic substrates has been reported in *Streptomyces lividens* (Harchand and Singh, 2001). In the present study, natural substrate in the form of apple pomace caused induction of FPase in *Bacillus macerans*. Notably the growth on apple pomace supplemented with CMC and avicel resulted in higher xylanase and FPase levels, then on apple pomace alone. This might result from cooperation between various inducers derived from the component of apple pomace, CMC and avicel.

As in the present study (Table 17), 10 per cent inoculum size of *Bacillus subtilis* was reported earlier (Krishna and Chandrasekran, 1996) to produce maximal amylases on

banana waste in solid state fermentation and production of alkaline protease by *Bacillus licheniformis* S-40 (Sen, 1995). Archana and Satyanarayana (1998) observed that maximum xylanase production was obtained with 2% inoculum size in submerged fermentation. Inoculum size control the initial lag phase of microorganisms producing cellulases. A smaller inoculum size extends the lag phase whereas larger inoculum size increased the moisture content to a significant extent. The free excess liquid present will give rise to an additional diffusional barrier together with that imposed by solid nature of the substrate and lead to increase in growth and enzyme production (Muniswaran *et al.*, 1994). Observations of the effect of inoculum size on maximal enzyme production in the present study agree well with the facts stated above.

A consistency between the factorial experiment and in the individual experiments for the conditions that gave maximum xylanase and FPase activity has not been observed as is clear from the results in two way tables of xylanase and FPase activity (Tables, 19,20,21,22). As much as 2.5 times increase in xylanase activity is obtained when the cumulative effect of the optimized conditions is taken into account. FPase activity was not affected much by these conditions. The conditions that gave maximum cell number also gave the highest xylanase activity. The data indicate that the xylanase and cellulase production is closely related to number of cells present. This, however, is not a suprising finding since apple pomace is highly biodegradable and serve as a good substrate for growth of the organism. The factorial method and the completely randomized design (CRD) is a statistical approach that examines all the possible combinations of independent variables at appropriate levels in single experiment. Testing of statistical significance of main effects with two factor interaction and four factor interaction have been discussed separately with the help of two way tables wherever necessary for the concerned factor. This approach guarantee faster achievement of optimal conditions with fewer nutrients at say two concentrations and with minimum cost and minimum time, thus rendering the comparison valid. It is certainly impractical for large number of variables.

Although it is difficult to compare yields reported in the literature because of the variation in the methodology of the assay. However, the enzyme yield can be compared to each other based on the measurement of the micromole of reducing sugars released per minute under the assay conditions. The xylanase yield reported by various workers in solid state fermentation for *Bacillus* strain include 97 Ug^{-1} DBP (Satyanarayana, 1994), 15 Ug^{-1} DBP (Archana and Satyanarayana, 1997) with *Bacillus subtilis* and *Bacillus licheniformis*, respectively and FPase yield reported by various workers in solid state fermentation is 4.27 Ug^{-1} (Muniswaran and Charyulu, 1994), 6U (Shamala and Sreekahtiah, 1986) with *Trichoderma viride* NCIM 1051 and *Aspergillus ustus*. The present isolate of *Bacillus macerans* could produce (1329 Ug^{-1} DBP) of xylanase under the optimum conditions used. Above all, it takes only 72 h to achieve maximum xylanase and cellulase production. Thus with reduced batch time, cheap substrate in the form of apple pomace and higher concentration of product formed, the use of solid state fermentation technique for bacterial xylanase production will lead to considerable reduction in capital investment.

SUMMARY AND CONCLUSION

Chapter-6

SUMMARY AND CONCLUSION

During the present investigation, attempts were made to find out the amenability of *Bacillus macerans* to produce extracellular xylanases and cellulases on apple pomace in solid state fermentation. The culture parameters for optimum enzyme production and effect of additional carbon and nitrogen sources was investigated. The organism was able to grow extensively on apple pomace and growth was enhanced when apple pomace was moistened with basal salt yeast extract medium (BSYEM).

The alkalophilic *Bacillus macerans* strain was found to be efficient producer of xylanase activity (535 Ug^{-1} DBP) but not a good producer of cellulase (FPase) activity (0.972 Ug^{-1} DBP). Other cellulase activities i.e. CMCase, avicelase and β-glucosidase were not detected. The production of xylanase and cellulase reached a peak in 72 h. Temperature (35°C), pH (8.0), cooking time (121±1°C) and inoculum size (10%) was found to be optimum for both FPase and xylanase production.

The studies on the effect of different carbon and nitrogen sources and other additives on the xylanase and cellulase production revealed that optimum xylanase and cellulase production could be achieved at a concentration of 0.5% (w/v) yeast extract and 3 mg% (w/v) $CaCO_3$.

The combined effect of pH, inoculum size, yeast extract and carboxy methyl cellulose on cellulase and xylanase production in 2^4 factorial experiment resulted in 2.5 fold increase in the xylanase activity, whereas FPase activity was decreased. A maximum xylanase activity (1329 Ug^{-1} DBP) and FPase activity (1.28 Ug^{-1} DBP) was found when fermentation was carried out at 35°C with fixed substrate concentration of 10 g apple pomace, pH 8.0, 3 mg% CMC and initial inoculum size of 10% (v/w). Thus, the

optimum xylanase activity in the factorial experiment was obtained when the conditions that gave maximum xylanase activity in the individual experiments were combined. However, this was not true for production of FPase activity.

On the basis of the results of present study, it is concluded that utilization of apple pomace as solid substrate in solid state fermentation could lead to large scale production of xylanases and also contribute to safe and economic waste management of apple pomace in the environment, where this waste is continuously accumulating and causing serious pollution problems.

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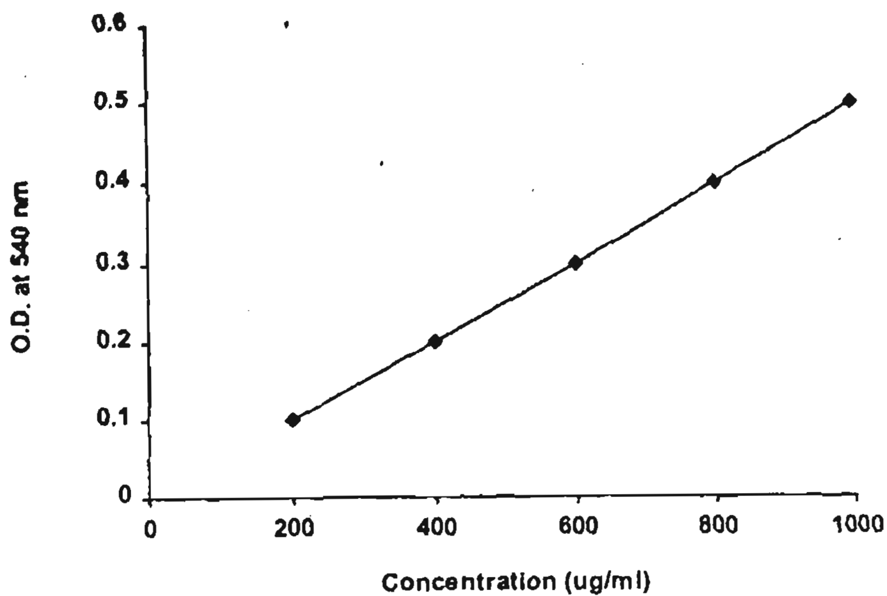
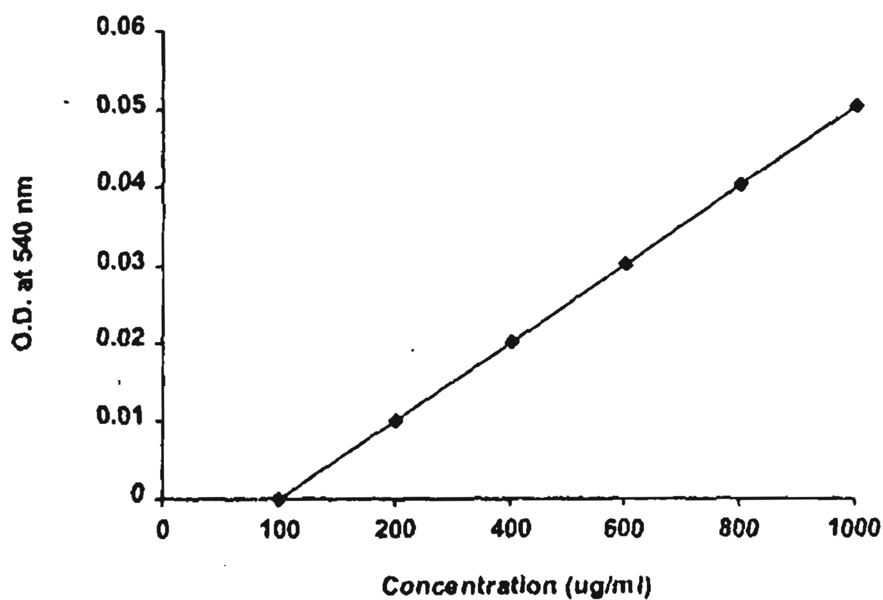
* - Original not seen

APPENDIX

APPENDIX-I

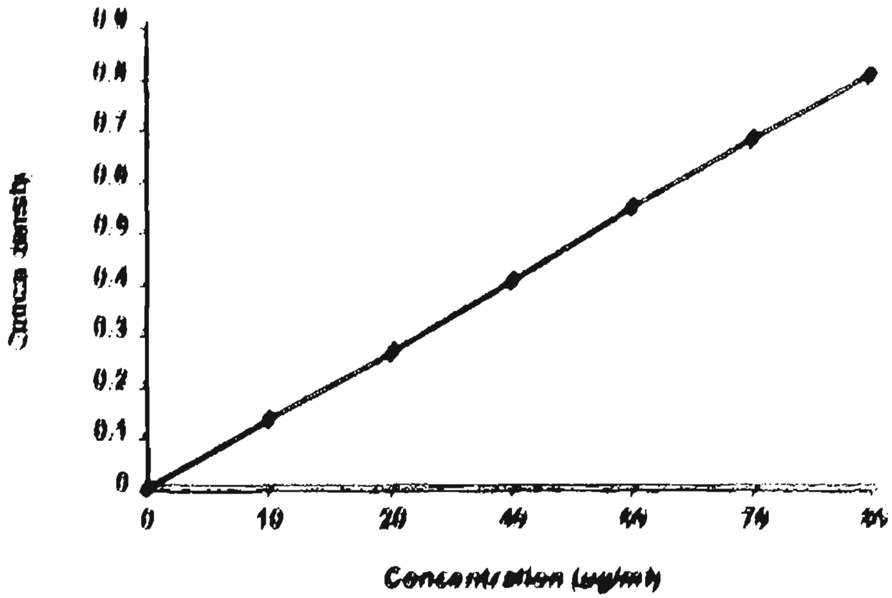


Standard curve of xylose



Standard curve of glucose

APPENDIX-II



Standard curve of p-nitrophenol

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THESIS ABSTRACT

Title of Thesis	:	**Apple pomace as a substrate for cellulase production by *Bacillus macerans* under solid state fermentation**
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Major Field	:	Microbiology
Minor Field(s)	:	i) Biochemistry
Degree Awarded	:	M.Sc. Microbiology
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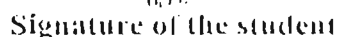
ABSTRACT

Solid state fermentation refer to the growth of microbes on solid substrates without the presence of free flowing liquid. During the present investigation, an attempt was made to find out the amenability of *Bacillus macerans* to produce extracellular xylanase and cellulase on apple pomace in solid state fermentation. The culture parameters for optimum enzyme production and effect of additional carbon and nitrogen sources was investigated. The alkalophilic *Bacillus macerans* was found to be efficient producer of xylanase activity (535 Ug^{-1} DBP) but not a good producer of cellulase (FPase) activity (0.972 Ug^{-1}). Other cellulase activities i.e. CMCase, avicelase and β-glucuronidase were not detected. Temperature (35°C), pH (8.0), cooking time (121±1°C) and inoculum size (10%) was found to be optimum for both FPase and xylanase activity. The combined effect of pH, inoculum size, yeast extract and carboxy methyl cellulose on cellulase and xylanase production in 2^4 factorial experiment resulted in 2.5 fold increase in xylanase activity whereas FPase activity decreased. A maximum xylanase activity (1329 Ug^{-1} DBP) and FPase activity (1.28 Ug^{-1} DBP) was found when fermentation was carried out at 35°C with fixed substrate concentration of 10 g apple pomace, pH 8.0, 3 mg % CMC, initial inoculum size (10%).

Major Advisor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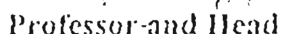
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