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CALLUS PRODUCTION EMBRYOGENESIS AND REGENERATION OF PLANTS IN SOME LOCAL VARIETIES OF RICE

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ABSTRACT

The plant matter was separated from the three varieties of Basmati rice (Basmati 2000, Basmati 385, and Super Basmati) to achieve one of the key goals. The seeds were surface sterilized, then inoculated with 2, 2, 5, and 3.5 mg/L of 2, 4-D on N6 medium to observe what would happen. The greatest incidence of callus acceptance recurrence and the fastest development, after just three weeks, were seen in the medium containing 2 mg/L of 2,4-D. Basmati 2000 performed best among the three varieties in terms of callus acceptance and plant recovery. It was observed that the callus aesthetic permeated all three collections. It was possible to tell apart the immobile, non-embryoid calli from the densely packed, massive embryoid calli. Over the course of three weeks, we gradually replaced the calli with maintenance medium (N6). Both 6-benzyl aminopurine (6-BAP) and naphthalene acidic corrosive (NAA) forms of basic salts and vitamins were usable in the setting of MS. When it came to revitalizing plants, the Basmati 2000 mixture performed the best, followed by the Basmati 385 and Super Basmati mixes. The calli were vaccinated and then put on recovery media, and after three weeks, roots began to develop. The growth-promoting effects of both BAP and NAA-containing media were seen, although none was enough for full recovery on its own. Callus acceptance was highest in all three varieties when grown in N6 medium with 2 mg/L of 2, 4 D. When grown on MS medium supplemented with 1 mg/L of NAA and 2 mg/L of BAP, however, Basmati 2000 and Super Basmati produced much better yields. Treatment with 1 mg/L of NAA and 5 mg/L of BAP resulted in the greatest recovery in the Basmati 385 variety.

INTRODUCTION

The pursuit of genetic variety in rice, a staple crop of significant importance in developing nations, has the potential to provide widespread access to the advantages offered by scientific and technological advancements. This might positively impact the lives of hundreds of millions of people in need. The advancement of cellular research and atomic breeding technology has been associated with the genetic enhancement of rice. The dissemination of non-native rice species has been associated with the use of tissue culture methodologies such as anther culture, advancements in life support systems, and the utilization of somaclonal variants.

Plants have the ability to undergo rejuvenation via mechanisms known as prolonged embryogenesis or organogenesis. In order to modify a plant, the use of in vitro tissue culture techniques may be necessary. In order to cultivate a whole plant inside a laboratory setting, it may be necessary to establish distinct evidence of living cells or tissues.

This research examined the effects of excessive moisture on three commonly cultivated plant species in the Punjab region. Shimada et al. (1969) and Vasil (1982) conducted studies on tissue culture challenges pertaining to rice cultivation in Pakistan. However, it is worth noting that no study has been conducted so far on the specific varieties developed in the region of Punjab. Regenerative rice cultures have been established with several forms of explants, including developed seeds, scutellum, and root-activated callus. The study demonstrated that there are significant variations in the formation and healing process of calluses across individuals.

For many decades, scholars in the disciplines of genetics, botany, and plant pathology have used callus and cell samples, alongside other plant components such as stems, flowers, roots, and eggs, in their research endeavors. This phenomenon might be attributed to the pluripotent nature of plant cells, which enables them to differentiate into many specialized cell types within the plant organism. The remarkable flexibility and capacity for growth from many cell types are retained by plants when cultivated in a laboratory setting. This phenomenon may arise due to physiological, physical, or restorative causes. Schwann (1939) concurred with the notion that every cell within a multicellular organism had the capacity for autonomous growth, provided it is provided with suitable conditions. The term "totipotent cell" was used by Morgan in 1901 (Krikorian &Berquam, 1969) to designate a solitary cell with the capacity to develop into a whole organism.

The present discourse aims to provide a concise overview of the historical development of tissue culture techniques specifically applied to the cultivation of cereal crops. 2.1

Larue (1949) is credited with pioneering the successful cultivation of corn endosperm. Carew and Schwarting (1958) as well as Roberts and Road (1955) both successfully generated a viable callus from mature rye plants. Norstog's research on community development behavior (Norstog, 1967, 1970) expanded upon his earlier study conducted in 1961. The research conducted by Yamaguchi et al. (1970) used wheat embryos as a model system to investigate the acceptance of callus and subsequent separation and development of cells in suspension.

2.2 Tissue Culture Utilizing Rice

The first attempts to cultivate rice using juvenile hybridized eggs and isolated root organ cultures were conducted by Fujiwara and Ojima in 1955, followed by Amemiya et al. in 1956. Since the 1960s, Japanese researchers have been endeavoring to develop methods for cultivating rice without the use of traditional seeds. The first effective callus formation was achieved by Furuhashi and Yatazawa (1964) by the use of rice stem hubs. Subsequently, the authors emphasized the significance of yeast extract in their study (Yatazawa et al., 1967). The approach used by Yamada et al. (1967b) was utilized for the cultivation of rice calli inside a laboratory setting. The study conducted by Yamada et al. (1967a) revealed that the application of indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) positively influenced the

formation of callus in base and shoot node 3. Plants were successfully regenerated by the use of several callus procedures, including seed callus (Tamura, 1968; Nishi et al., 1968), root callus (Kawata and Ishihara, 1968), and dust callus (Niizeki and Oono, 1968).

In this study, coleoptile fragments derived from Infica rice (namely, the Kasturi variety) were used in an effective methodology aimed at achieving a high frequency of acceptance of embryogenic callus and subsequent plant regeneration. The coleoptile pieces were utilized at various developmental stages, specifically 3, 4, 5, and 7 days post-germination.

The coleoptile sections were cultivated in MS basal medium supplemented with 2,4-D (4.50-18.0 M), kinetin (2.32 mu M), and 3% sugar. As a result, the cut ends of the sections underwent a transformation, forming a rigid callus. Embryogenic calli were cultured in a Murashige and Skoog (MS) medium supplemented with a concentration of 2.25 millimolar (mM) 2, resulting in the development of viable embryos. The use of a medium containing a 4-dimensional structure, a concentration of 2.32 millimolar kinetin, 490 micromolar L-tryptophan, and 3% (w/v) sugar resulted in a notable increase in callus acceptance and substantial fetal development. According to Sahrawat and Chand (2001), The embryogenic callus clumps were transferred to a Murashige and Skoog (MS) medium supplemented with 2.85 micrograms per liter (mu M) of indole-3-acetic acid (IAA), 17.77 mu M of 6-benzylaminopurine (6 BAP), and 3% (w/v) of sugar. Consequently, this resulted in the regeneration of plant life.

In their study, Gul et al. (2000) examined the response of four rice varieties, namely Basmati-385, JP-5, Pakhal, and Swat-Il, to various cultural circumstances in the context of in vitro callus organization and plant regeneration. The efficacy of culture conditions supplemented with 2 mg/L 2,4 D and 0.2 mg/L kinetin, as well as those devoid of these hormones, was assessed to determine their impact on callus growth. Pakhal (70%) and Basmati-385 (58%) exhibited significant callus elongation during cultivation on a growth hormone supplemented medium. For

The Kn levels used for recovery in the MS medium are MSK2 (2.0 mg/L) and MSK2 (0.2 mg/L). The experiment included the use of Kn and IAA at concentrations of 0.5 mg/L and MSKS at a concentration of 5 mg/L. The MSKS medium, namely MSKS medium (not MSK2), demonstrated superior performance across all genes in terms of wound healing. The therapeutic methods that exhibited the highest levels of cruelty were the JP-S type (64%), followed by Swat-II (51%), Pakhal (44%), and Basmati-385 (30%).

The topic of interest is rice cultivation in the Chorotega region of Costa Rica. In their study, Shankhdhar et al. (2001b) enhanced the development and proliferation of Pusa Basmati I embryos by a specific protocol. This included subjecting the embryos to a dark environment at a temperature of 26-41°C for a duration of four weeks. Additionally, the researchers supplemented the MS medium with certain components, including 2 mg/L of 2,4-D, 3% sucrose, and 8 g/L agar. The concentration of 2,4 D in the medium decreased as the fetal callus developed from the developing embryo. The manipulation of the MS-IAA-6BAP and kinetin genes in the eggs

resulted in the development of robust and vigorous plants. When exposed to a concentration of 0.5 mg/L of BAP, the plantlets exhibited optimal self-arrangement.

The study conducted by Biswan and Mandal (1999) investigated the behavior of several varieties of in vitro-cultivated indica rice with regard to callus acceptance and plantlet healing. The obtained findings were compared to those of Taipei 309, a strain of japonica rice that exhibits robust growth in a laboratory environment. Annada extracted the highest number of plantlets from each seed call, and subsequently obtained the greatest number of plantlets.

The plant species known as Oryza sativa L. has the ability to undergo callus formation. cv. The present study investigated the impact of 2, 4D alone (at pH 0 and 1) and in conjunction with BAP (at a concentration of 0.5 g/L) on Super Basmati seeds. Following fertilization, the compact calliwere transferred to a growth medium for a duration of four weeks, after which they were then transferred to a healing media. The concentrations of NAA and BAP used for the purpose of recovery ranged from 0 to 0.1 mg/L and 10 mg/L, respectively. The frequency of callus formation in Super Basmati varied from 54.6% when cultured on N6 medium to 87.7% when cultured on MS media. A marginal enhancement was seen in the proliferation of the callus.

N6 has a moderate level of effectiveness when compared to MS. When examined in isolation from the conventional media, it was shown that the application of 2,4-D at a dosage of 2 mg/1 yielded the highest rate of callus development and acceptance. According to Rashid et al. (2001), the inclusion of BAP and 2,4-D in the callus acceptance medium resulted in a significant reduction in the rate and frequency of callus acceptance.

The objective of this research endeavor was to enhance an existing technique that has previously shown the ability to induce callogenesis in rice. The experiment included three distinct varieties of rice, namely Rachna Basmati, Basmati 2000, and 370. The highest callogenesis was seen in Basmati 370 when cultivated on N6 medium supplemented with 2, 4-Dic 1.2, and 3mg/L of the hormones. In the process of callogenesis, no discernible difference was seen between Rachna Basmati and Basmati 2000. The callus acceptance rate of Basmati 370 was shown to be decreased in comparison to Rachna Basmati, Basmati 2000, and Basmati 370. The study conducted by Rashid et al. (2003) examined the development of callus in three different varieties, with Rachna Basmati demonstrating the most favorable results.

To further investigate soma-clonal variation in two varieties of rice (Oryza sativa L), a single round of sexual reproduction was conducted on R-0 rescued plants, sandwiched between two cycles of cultivating callus cultures obtained from embryos. Following a single round of tissue culture, the regenerated plants exhibited diminished regularity and aesthetic quality compared to the initial stock. By using R-1 embryos as explants in a subsequent cycle, it seemed that the heritable trait of cellular salt resistance shown in the first cycle might be transmitted to the offspring. The extent and kind of somaclonal variation is contingent upon the characteristics of the R-0 parental plant and the specific circumstances of the culture environment (Lutts et al., 2001). Research findings have shown that the level of somaclonal diversity seen in certain lineages cultivated from calli subjected to salt treatment is significantly reduced.

This study used immature seed callus derived from four distinct varieties of Australian rice to investigate the regenerative potential of plants within a laboratory setting. The manner in which the media portrays a state of unyielding acceptance.

The strains exhibited the ability to cultivate calluses when exposed to varying concentrations of 2,4-D in MS medium, ranging from 0.5 mg/L to 2.0 mg/L. The recently regenerated shoots were placed in a growing mixture without the typical plant development nutrients. The discovered plant specimens exhibited a wide range of common and diverse morphologies.

According to the study conducted by Azria and Bhalla in 2000, According to our study findings, callus derived from the cultivation of several rice embryo types has sufficient flexibility to be used in genetic modification studies.

In their work, Nouri and Arzani (2001) used MS, LS, and N6 medium to investigate the callus acceptance and plant regeneration potential of 18 distinct rice genotypes derived from immature eggs. The measurement of plant healing rate was conducted by transferring the calli from the acceptance medium to the recovery medium. Due of its rapid regrowth, Japonica rice emerged as the predominant variety for cultivation. According to the findings of this research, MS and N6 media were identified as the most suitable growth mediums for cultivating rice seedlings in a laboratory setting.

The seeds of Basmati 370, Basmati 385, and KS 282 rice varieties were cultivated on a growth medium consisting of MS medium supplemented with 2.0 mg/L 2,4-D. Among the tested plant varieties, KS 282 had the greatest success rate (33.0%) in callus formation, followed by Basmati 385 (17.6%) and Basmati 370 (6.5%). Various combinations of auxin and cytokinin were used to facilitate the transfer of calli from Murashige and Skoog (MS) medium. The plant regeneration was seen to be maximum after a period of 90 days of maintenance when the MS medium was supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP for Basmati 370, resulting in a regrowth rate of 57.14% for Basmati 385. In a study conducted by Rashid et al. (2003), it was shown that KS 282 exhibited the highest recovery rate of 75% when subjected to lesser dosages (0.4 mg/L NAA and 0.8 mg/L BAP) on the same medium.

A research was conducted using three indica rice (Oryza sativa L.) varieties to investigate the impact of genetics on callus acceptance and the recovery of green plantlets in response to different medium combinations.

The Kamal District includes the surrounding areas including IR-72 and IR-54. A total of 15 distinct media combinations were used in order to assess the scutellar callus inference in developing embryos that had undergone incision. The combinations included MS, N6, R2, SKI, and other derivative forms. The acceptance of callus, as well as its subsequent recovery, is influenced by several factors including the acceptance medium, the recovery medium, and the combinations of genotype, media, callus acceptance, and recovery. Additionally, the interactions between genotype, recovery, callus acceptance, and medium may also impact the acceptance and

recovery processes. The Kamal region had the best percentage of recovery (47.5% in IR-72) when the callus-inducing agent SKIm was synergistically paired with the recovery-enhancing medication MS. A significant proportion of individuals who had MMS(N)-MMS(N) therapy prior to the administration of IR-54 (25%) exhibited successful regeneration. Although genetics and the composition of the basal medium used for callus acceptance were significant factors contributing to the recovery response, a comprehensive examination of variance indicated a crucial interaction between the media used for callus and plantlet recovery (Khanna and Raina, 1998).

Scholars have conducted investigations on the genetic mechanisms behind the regenerative capacity of the seed callus in the resilient rice cultivar known as Joshu. An experiment was conducted to assess the regenerative capacity of the progeny resulting from a cross between Joshu and two cultivars known for their ability to heal from injury, namely Moritawane and Norin 1. The findings indicate that the tall recovery ability seen in Joshu is governed by two distinct factors: a dominant character and a latent character. High recovery capacity is shown when either one or both of these features are exhibited. Furthermore, this research could not identify any discernible disparities in aptitude between different generations. (2021). The Impact of Social Media on Mental Health: A Review of the Literature)

Scientists have used groome change, chromosomal transformation, and plasmon transformation techniques to assess the somatic diversity of offspring plants belonging to the R-0, R-1, and R-2 generations.

The progeny of R-2 plants exhibited the largest proportion (52.4%) of quality modifications, with albinos and other chlorophyll-deficient mutants accounting for a range of 20 to 33. The study demonstrated that callus cells exhibit a significant variation in chromosomal count, which is influenced by the aging process. According to Chatterjee (1998), the findings indicated that the use of tissue culture techniques may result in a substantial occurrence of somaclonal variation.

The effects of isopentenyl adenine (2ip) on wheat tissue and plant healing were investigated by researchers by cultivation of the chemical under several environmental conditions. The embryogenesis and plant recovery rates of rice calli were significantly enhanced upon transfer to a conventional tissue culture recovery medium, subsequent to preculturing at certain 2ip ratios. The inclusion of 2ip in the preculture medium significantly influenced the subsequent stage of cell separation in the context of 2,4-D assistance. By altering the concentration of 2,4-D and 2ip in the growing media, it is possible to differentiate the processes of plant development and plant healing. Zhu et al. (1996) provide several methodologies for the use of 2ip, 2, 4-D, and KT in the context of grain recovery.

on this study, grain and root explants were used on a Murashige and Skoog (MS) medium to assess the regenerative capacity and healing ability of nine indica rice varieties and one indica-cross line. The outcomes seemed to be contingent upon an individual's genetic makeup. The recognized percentage of callus healing in corn cultivation increased significantly from 24% to 93.3%. According to the study conducted by Rahim et al. (1991), the plant regeneration rates observed in plants using grain calli varied between 6.2% and 18.6%.

The study included the cultivation of callus societies derived from 7-week-old embryos of several indica rice varieties. The morphogenic calli rates observed ranged from 10% to 47%. It was observed that these plants exhibited the ability to undergo healing processes.

We conducted experiments involving callogenesis and plant resurrection using three distinct growth media. The use of Murashige and Skoog basal media, namely MSC for callogenesis and MSR for recovery, proved to be the most effective medium combination among the seven types evaluated for plant revival. The rates of callogenesis were not commensurate with the regenerative capacity of plants. The CR-1113 and CR-5272 lines had the highest levels of greenness among the plants. According to Valdez et al. (1997), the findings of their research indicate a potential correlation between genetic variations in several plant species and their capacity for regeneration.

The investigation included an examination of the morphological characteristics of rice (Oryza sativa L.) callus cultivated in a laboratory setting, as well as an assessment of the regenerative capacity of the plant from this callus tissue. A model was developed to characterize the many morphological characteristics shown by rice calluses. In a study conducted by Kucherenko (1993), a total of 33 rice calluses were collected and visually shown via drawings. Subsequently, these calluses were categorized into four distinct categories according to their respective healing capacities.

MATERIALS AND METHODS

The study's main objective was to develop and analyze greenhouse-grown rice callus samples. The probe took place at ABP. I'll be visiting the National Agribusiness Research Center in Islamabad between December 2022 and January 2023.

ORIGINAL SOURCE

Islamabad's NARC's Plant Breeding Program is where we got our seeds for the Basmati 2000, Basmati 385, and Super Basmati varieties of rice.

PROCEDURE FOR STERILIZATION

The sterilization procedure described by Rashid et al. in 1996 involves cleaning dishware and sanitizing the explant's exterior.

Explant Sterilization

A laminar flow cover was used throughout the explant sterilization process to ensure that no contamination might occur. We autoclaved water in carafes and dropped precisely chosen sound seeds within. Seeds were soaked for one minute in 70% ethanol before being rinsed in purified water that had been heated in an autoclave. After soaking the seeds for 20 kilometers in a 50% clorox solution, they were vigorously shook for the whole duration. Clorox is a commercial bleach that has 5.25 percent sodium hypochlorite in it. The Clorox treatment was followed by

three thorough rinses in filtered water from an autoclave. The seeds were allowed to germinate, and then moved to a petri dish using sterile filter paper.

Forming Content for Media

A combination of salts and vitamins from Murashige and Skoog (1962) and Chu (1978), as well as sucrose and plant growth regulators, served as the basis for the callus refining protocol. Table 1 displays the current inventory status of MS and N6 media components.

Table No.1: The Individual Parts That Make Up MS and N6 MediaMS(mg/L) N6(mg/L)

No.	**Components**	**MS(mg/L)**	**N6(mg/L)**
1.	**Macronutrients(A stock solution)**		
	Ammonium nitrate	1650	-
	Potassium nitrate	1900	2830
	Calcium chloride	370	185
	Magnesium sulphate	170	400
	Monopotassium dihydrogen phosphate	-	283
2.	**Micronutrients (B stock solution)**		
	Boric acid	6.2	1.6
	Cobalt chloride.6H_2O	0.025	-
	Copper sulphate	0.025	-
	Mangnese sulphate. H_2O	15.6	3.3
	Iron sodium chelate	43	43
	Potassium iodide	0.83	-
	Sodium molybdate	0.25	0.25
	Zinc sulphate. 7H_2O	8.6	1.5
3.	**Vitamins (C stock solution)**		
	Thiamine- HCL	0.5	10
	Pyridoxin- HCL	0.5	0.5
	Nicotinic acid	0.5	0.5

	Myo- inositol	100	-
4.	**Carbon source(sucrose)**	**30g/L**	**30g/L**

1. Macronutrients (Readily Available). The ratio of ammonium nitrate to potassium nitrate is 1650:1900:2830.370 mM Calcium Chloride 185 mMSulfate of magnesium, 170-400Dihydrogen phosphate, monopotassium - 283

Ingredients for B Stock Solution, MicronutrientsSodium borate6.2 1.6 Chloride of cobalt.Sulfate of copper (CuSO4) and manganese (MnSO4), each at 0.025 percent in 6H2O. H2O 15.6 3.3 Chelated iron (sodium) 43The ratio of potassium iodide to sodium molybdate is 0.83 to 0.25. Sulfate of zinc. 7H2O 8.6 1.5

Vitamin C (as a stock)HCL Thiamine.5 HCL Pyridoxin.5 HCLHalf a half of nicotinic acid Inositol 100 Myo-Sugar, the fourth carbon source30g/L 30g/L.

Disc sterilization

Wrapped test tubes containing ober surgical rebellious and distilled water are autoclaved at 15 pressure for 20 minutes at 121 °C.

INDUCTING CALLUSES

Seed Inoculation One seed was placed in each test tube. Societies were able to interact and advance in a natural climate-controlled area. They were strung vertically, 10 inches apart, from regular electric fluorescent lamps that produced a steady, strong light of around 2000 lux. The environment was kept at 25 degrees Celsius (3 degrees Fahrenheit) for the duration of the development period. After vaccination, callus acceptance was restored in all genotypes within four to five days. All varieties were tested for callus development rate and quality two to three weeks after vaccination. Method of Trade and Cultural Infiltration

To assure the cleanliness of the swabs, we moved them inside a laminar-flow cabinet. The calli were collected in Petri plates, and the embryogenic callus was then cultured in a maintenance medium similar to the callus acceptance media. For three weeks, the institutions depended on media created for upkeep.

REGROWTH OF PLANTS

Murashige Skoog's (1962) salts, vitamins, and 3.0 percent (w/v) sucrose were used to assess the plant recovery potential of four genotypes. Six-benzyl amino purine (BAP) and indole acidic corrosive (IAA) were used in two different combinations as growth regulators. Carefully separating embryogenic (E) calli from non-embryogenic (NE) callus, the latter were then divided into smaller pieces and inoculated on the recovery medium shown inside the glass vials, resulting in a complete plant recovery. The recovery medium, at a volume of 50 ml each combination, was individually poured into glass containers measuring 55 mm in diameter and 120 mm in depth. In each shock, around four or five calli received a cap immunization. For a period of two to three

weeks, the test tubes and jars were stored in an environment similar to that required for callus acceptance.

BAP and NAA make up the two combinations tested for their efficacy as a recovery cocktail (Table No. 2).

No.	Conc. of NAA (mg/L)	Conc. of BAP (mg/L)
1.	1	2
2.	1	5

NAA and BAP Concentrations in Two Different MS Media

No. NAA Concentration (mg/L)BAP Concentration (mg/L)

1. 1 2 2. 1 5

These were used in conjunction with MS salts and served in 2% sugar arrangements.

3.4 BECOMING A GLASS HOUSE

First, all of the plants that had been rescued were moved into the greenhouse, where the humidity and temperature could be carefully maintained. As time went on, the temperature adapted progressively to its new surroundings. After these plants hardened, they were transplanted into the wild, where they thrived despite the fact that no one knew whether or not they were disease-free (Figs. 7, 8, and 9).

RESULT AND DISCUSSION

The objective of this study was to ascertain the most effective combination of the growth regulators benzyl amino purine (RAP) and naphthalene acidic corrosive (NAA) in order to enhance the regeneration process of three distinct varieties of rice (Oryza aiva 1): Basmati 385, Basmati 2000, and Super Basmati.

Prior to commencing tissue culture tests, it may be necessary to sterilize the explants. In their study, Rashid et al. (1996) provided a description of the sterilization procedure used in this inquiry. The researchers utilized a combination of 70% ethanol and 50% clorox to sterilize the rice seeds. The most pronounced rate of callus development was seen when a 50% concentration of Clorox was used. Li, et al. (1992) conducted a study. According to the available research, seedlings subjected to a 30-minute treatment with Clorox 45% (v/v) were seen to exhibit germination.

The study conducted by Shankhdar et al. (2001a) investigates the role of 2,4-D in the induction of a callus. The available evidence from previous studies strongly indicates that the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) is a necessary need for the process of dedifferentiation in rice callus prior to its successful acceptance. Consequently, a solution of 2,4-D was administered to N6 medium including ure seeds of several kinds at concentrations of 2, 2.5, 3.0, and 3.5 mg/L. In a previous study conducted by Naheed Gul al (2000), it was shown that the inclusion of

growth hormone in the medium resulted in enhanced callus formation. Consequently, in the present study, plantlets were cultured on N6 medium without 2,4-D, as an alternative to callus cultivation.

This observation suggests that 2, 4-D exhibits effects on the genome, providing a possible explanation for the significant role of 2, 4-D in callus formation, as reported by Fan et al. (2000).

The greatest quality callus was seen when a concentration of 2 mg/L of 2,4-D was used. Consequently, the presence of different concentrations of 2, 4-D led to alterations in the quality of the callus. The callus acceptance rates for Basmati 2000, Super Basmati, and Basmati 385 were found to be 85%, 80%, and 60%, respectively. According to Rashid et al. (2001) and Shankandar et al. (2001b), the application of 2,4-D at a dose of 2 mg/L on MS medium yielded the most favorable rates of callus acceptance in rice. Nevertheless, as shown by the data presented in Table 3, higher dosages of the substance were found to impede the process of cell division. The frequency of the three accepted calluses exhibited significant variation due to the distinct genetic ability of each type to absorb calluses (Schaeffer et al., 1979). Among the several varieties tested, the Basmati 2000 shown considerable promise, but the Basmati 385 variety did not demonstrate similar potential. The consistency of the callus's appearance is evident across many breeds, as seen in Figures 1, 2, and 3. The promotion of cell division and proliferation is attributed to the auxin properties of 2,4-D. The process of mRNA and protein conjugation is subject to external influences. Hence, it is plausible that a concentration below the ideal range would not sufficiently permeate the combination of mRNA and proteins, but a quantity beyond the optimal range might potentially intensify the alteration (Malik and Srivastava, 1985).

Table No. 3. Effects of different concentrations of 2, 4- D on callus induction.

2,4- D Concentration (2 mg/L)	Callus Induction Frequency (%)		
	Basmati 2000	Basmati 385	Super Basmati
2.0	85.0	60.0	80.0
2.5	81.0	54.5	75.0
3.0	74.2	51.7	69.0
3.5	70.0	50.0	67.5

Table No.4. Callus induction frequency of Super Basmati on N6 medium with 2 mg/L 2,4- D

No. of Replicates	Total seeds culture	Callus Indeed	Percentage Frequency (%)
1.	120	90	75
2.	96	70	72.9

3.	144	103	71.5
4.	144	105	73

Mean = 73.1%

Table No.5. Callus induction frequency of Basmati 385 on N6 medium with 2 mg/L 2,4- D

No. of Replicates	Total seeds culture	Callus Indeed	Percentage Frequency (%)
1.	72	37	52
2.	144	73	57
3.	96	42	55
4.	144	72	51

Mean = 53.75%

Table No.6. Callus induction frequency of Basmati 2000 on N6 medium with 2 mg/L 2,4- D

No. of Replicates	Total seeds culture	Callus Indeed	Percentage Frequency (%)
1.	168	129	76.78
2.	144	126	87.5
3.	120	105	87.5
4.	144	102	70.8

Mean = 80.6%

Table No.7. Comparison of Average Callus Induction (%) of Basmati 2000, Super Basmati and Basmati 385.

Variety	Average Callus Induction (%)
Basmati 2000	80.6
Super Basmati	73.1
Basmati 385	53.75

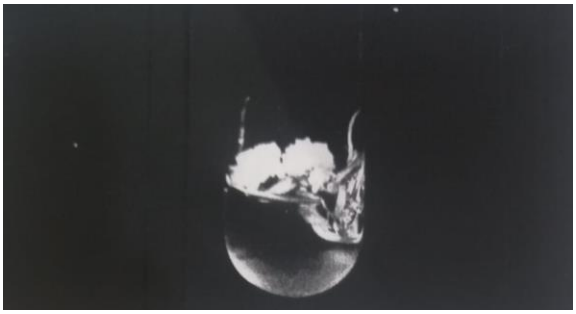

Fig. 1. Embryogenic Callus in Basmati 2000 on N6 at 2 mg/L of 2,4- D

Fig.2. Embryogenic Callus in Super Basmati on N6 at 2 mg/L of 2,4- D.

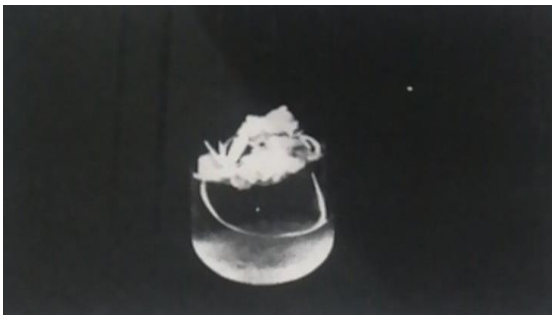

Fig.3. Embryogenic Callus in Basmati 385 on N6 at 2 mg/L of 2,4- D.

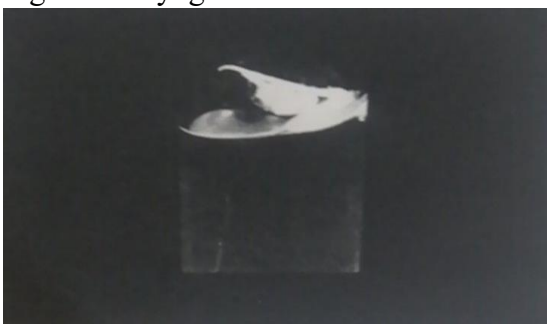

Fig.4. Non- Embryogenic Callus

Embryogenic and atypical calli are the two groups to compare.Calli were divided into embryogenic (EC) and non-embryogenic (NEC) groups according to their morphological characteristics. In Figs. 1, 2, and 3, you can observe the embryonic rice grains that gave rise to the EC calli, which have a compact spherical shape and a white to velvety look. When cultivated in the same N6 media, embryonic cells (ECs) may form structures that resemble, or are identical to, embryoid bodies. Friable, unrestrained calli were indicative of NEC (Fig. 4).

The process of callus reproduction is of interest

Important for a successful regeneration is the elimination of non-embryogenic or brown/dark sections of embryogenic callus. A 21-day period ended with the appearance of an embryoid. The best results for callus production and growth in the cultured medium were achieved when 2,4-D was used at a dosage of 2 mg/L. The varying mixtures all produced desirable results. Tables 8, 9, and 10 indicate the growth of the assortments used in the display displays. Katiyar et al. (1999) observed that the rate of callus proliferation differed from one genotype to the next. Genotypic uniqueness in the persistence of callus acceptance in Basmati cultivars was thoroughly described by Rashid et al. (2000). According to studies by Chen and Lin (1976) and Tsai and Lin (1977), the frequency with which a callus is accepted depends on the particular varietal type.

Table No.8. Maintenance of embryogenic calli of Basmati 2000 on N6 2 mg/L 2,4- D.

No. of Calli	Contamination	Browning	Growth and Proliferation	Growth(%)
25	4	3	18	72
40	6	5	30	75
40	4	7	29	72.5

Mean = 73%

Table No.9. Maintenance of embryogenic calli of Super Basmati on N6 2 mg/L 2,4- D.

No. of Calli	Contamination	Browning	Growth and Proliferation	Growth(%)
30	4	5	21	70
35	5	6	24	68.5
40	7	4	29	72.5

Mean = 70%

Table No.10. Maintenance of embryogenic calli of Basmati 385 on N6 2 mg/L 2,4- D.

No. of Calli	Contamination	Browning	Growth and Proliferation	Growth(%)
25	5	3	12	60
30	4	6	20	66
40	5	10	25	62.5

Mean = 62.8%

PLANT REGENERATION

On a modified Murashige and Skoog (MS) medium supplemented with 2 g/L of casine hydrolysate, 3% sugar, and 3% sorbitol, various varieties of calli were cultivated in anticipation of the harvest. Table 2 exhibits the two distinct NAA+BAP formulations utilized in the study. Both auxins and cytokinins played equally important roles in the regeneration of plantlets.The data presented in Table 7 pertain to the total number of green-spotted calli and the subsequent number of regenerated plants derived from these calli under varying BAP and NAA concentrations. Rashid et al. (2000) discovered that the close proximity of a high concentration of BAP had a positive effect on plant development. However, the experiment produced contradictory results, which may be attributable to the unique characteristics of the plant varieties utilized. All genotypes displayed significant variation in their recovery capacities. According to Kyungsoo et al. (2003), the lack of agar in the recovery medium had a negative impact on the growth of the seedlings. The initial allocation period of four days for verdant space has been extended to ten days. Basmati 2000 and Super Basmati exhibited recovery rates of 80.6% and 73.1%, respectively, when subjected to a standard treatment consisting of the application of 1 mg/L of NAA and 2 mg/L of BAP, according to the results of the experiment. These results are illustrated in Figures 5 and 4. In contrast to the results reported by Rashid et al. (2000), the application of 1 mg/L of NAA and 5 mg/L of BAP to Basmati 385 resulted in the greatest recovery to the initial level (Table 8, Figure 6). No one made the effort to observe the revival.

Initially, the seeds produced little root development; however, after four weeks, they had effectively established a solid foundation. Four weeks were required for the plants to reach the appropriate level of readiness for transplanting into the greenhouse environment. In addition, it is plausible that the presence of a sediment remediation expert would aid in the recovery process. The duration of recovery for calli immunized on an agar-based recovery medium is significantly longer than for calli immunized on a gel-based recovery medium.

Table No.11. Regeneration of calli in all the three varieties at 1 mg/L NAA and 2 mg/L BAP.

Variety	Total Calli Cultured	Calli Left after contamination	Blackening	Green Spots	Plants Formed	Percentage(%)
Basmati 2000	55	49	7	9	39	71
Basmati 385	60	50	18	8	20	33.33
Super Basmati	50	48	6	7	34	68

Table No.12. Regeneration of calli in all the three varieties at 1 mg/L NAA and 5 mg/L BAP.

Variety	**Total Calli Cultured**	**Calli Left after contamination**	**Blackening**	**Green Spots**	**Plants Formed**	**Percentage (%)**
Basmati 2000	40	36	4	6	24	60
Basmati 385	60	50	11	16	23	38
Super Basmati	38	32	10	6	22	57.8

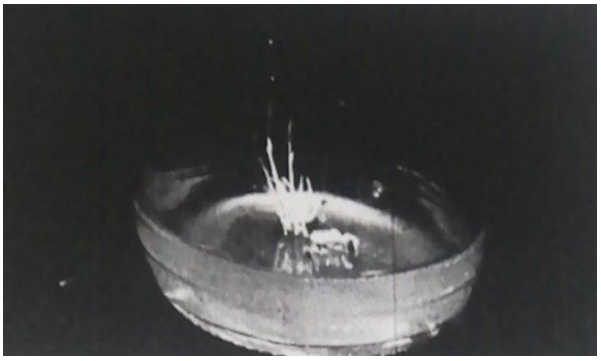

Fig.5. Regeneration of callus in Super Basmati

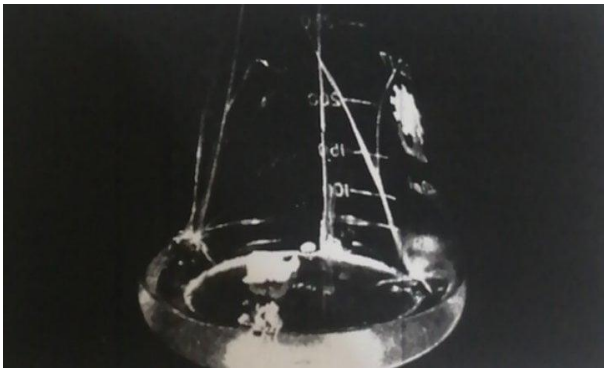

Fig.6. Regeneration of callus in Basmati 2000

Fig.7. Regeneration of callus in Basmati 385

GLASSHOUSE ASSESSEMENT OF THE PLANT REGENERATED FROM CALLI

In order to conduct a comparative analysis of the growth patterns shown by different plant species, a total of eight plants were transplanted inside the confines of the conservatory. The control plants of all cultivars propagated from seeds exhibited a significant level of development, but the tissue-refined plants had comparatively lower levels of development. There exist two potential rationales for this phenomenon: either the plants' heightened susceptibility renders them exceptionally amenable to the regulated environment of a greenhouse, or the plants first manifest reduced growth rates which subsequently escalate after they establish a stable foundation. The results of the study showed that the calli-grown plants of Basmati 2000 shown the highest level of growth compared to the other kinds examined. Conversely, the calli-grown plants of Basmati 385 demonstrated the lowest level of development. Certain specimens that have been retrieved exhibit a lack of growth, maybe due to an underdeveloped root structure that hinders their ability to collect essential water and nutrients from the surrounding soil. According to Su et al. (1992), the somaclones exhibited a somewhat limited growth in terms of height.

Table No.13. Glasshouse assessement of the somaclones of Basmati 2000

No. of Plants	Basmati 2000			
	Control		Somaclone	
	Height(cm)	No. of Tillers	Height(cm)	No. of Tillers
1	125	5	92.5	5
2	122.5	7	90	3
3	130	7	105	3
4	132.5	6	80	2
5	95	3	105	2
6	131	7	87.5	2
7	140	8	100	3
8	130	6	98.75	2

Table No.14. Glasshouse assessement of the somaclones of Basmati 385

No. of Plants	Basmati 2000			
	Control		Somaclone	
	Height(cm)	No. of Tillers	Height(cm)	No. of Tillers
1	75	4	27.5	1
2	80	5	32.5	2
3	62.5	3	13.75	1
4	72.5	3	17.5	1
5	52.5	0	27.5	1
6	0	4	12.5	2
7	20.5	2	0	0
8	72.5	3	8.75	1

Table No.15. Glasshouse assessement of the somaclones of Super Basmati

No. of Plants	Basmati 2000			
	Control		Somaclone	
	Height(cm)	No. of Tillers	Height(cm)	No. of Tillers
1	30	4	30	1
2	47.5	5	27.5	1
3	20	2	7.5	1
4	25	3	0	0
5	30	3	10	2
6	29	4	0	0
7	31	5	0	0
8	30	5	0	0

Fig.8. Growth of somaclone of Basmati 2000

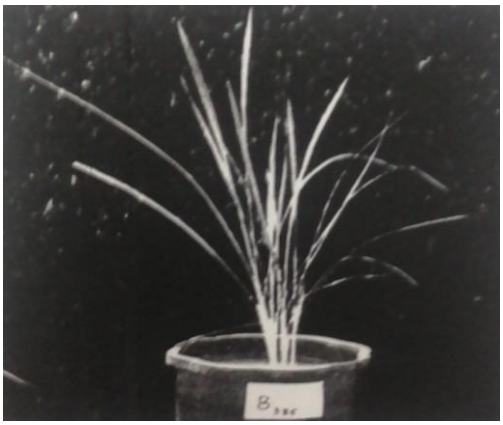

Fig.9. Growth of somaclone of Basmati 385

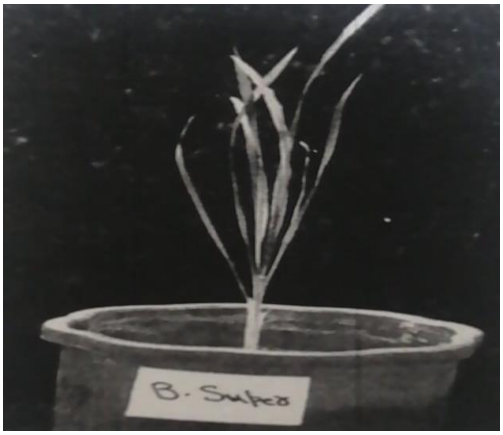

Fig.10. Growth of somaclone of Super Basmati

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PREVALENCE AND RISK FACTORS OF HEPATITISB IN TWIN CITIES

M. AITZAZ AKRAM, ZAHIDA NAWAZ, NADEEMARSHAD, MUHAMMAD BASIR SHAH, MUHAMMADIMRAN

ABSTRACT

Countries with poor economies tend to have higher rates of hepatitis B and C. Rawalpidi has a very high ratio (2.16), indicating a very type-specific language. At Bilal Hospital Rawalpindi, 2.16 percent of both replacement and voluntary blood donors were positive for HBV. Our study's results suggest to a rising prevalence rate, which, if not handled, might balloon out of control. Therapeutic injections, dental procedures, and barbershop shaving may all increase the risk of transmitting Hepatitis B Virus (HBV). It is suggested that public healthcare facilities switch from employing immunochromatographic tests (ICT) to using enzyme-linked immunosorbent assays (ELISA) to reduce the spread of Hepatitis B. It is also critical to educate the population at large about the prevalence of Hepatitis B in specific metropolitan regions.

INTRODUCTION

Classification

Hepatitis B virus' DNA origin was uncovered in the 1960s. According to the system established by the International Committee on Taxonomy of Viruses (ICT), this virus is in the family of orthopa DNA viruses known as Hepadnaviridae. Only one animal virus, from the spumaretrovirinae subfamily of the retroviridae family, uses viral RNA as an intermediate in the process of replicating its DNA genome. The estimated rate of hepatitis B transmission is 75-200 times greater than that of HIV. Hepatitis B is most often spread by skin-to-blood contact.

Hepatitis E, or HEV

Hepatitis B virus (HBV) has a lipid envelope and a spherical shape, with a diameter of 42–47 nanometers. As can be seen in figure 1.1, the virion consists of the nucleocapsid, the viral envelope, and an incomplete double-stranded DNA genome. The big L, small S, and intermediate M surface proteins make up the nucleocapsid core protein dimers, which were discovered in 2007 research by Seeger et al., 2007. Infected hepatocytes have a minus-strand covering the whole genome and a plus-strand covering around two-thirds of the genome.

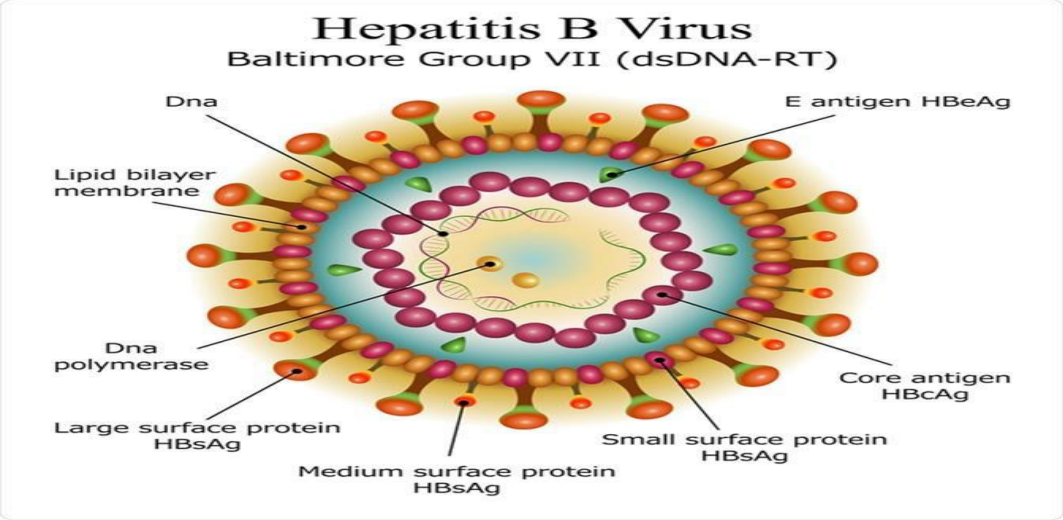
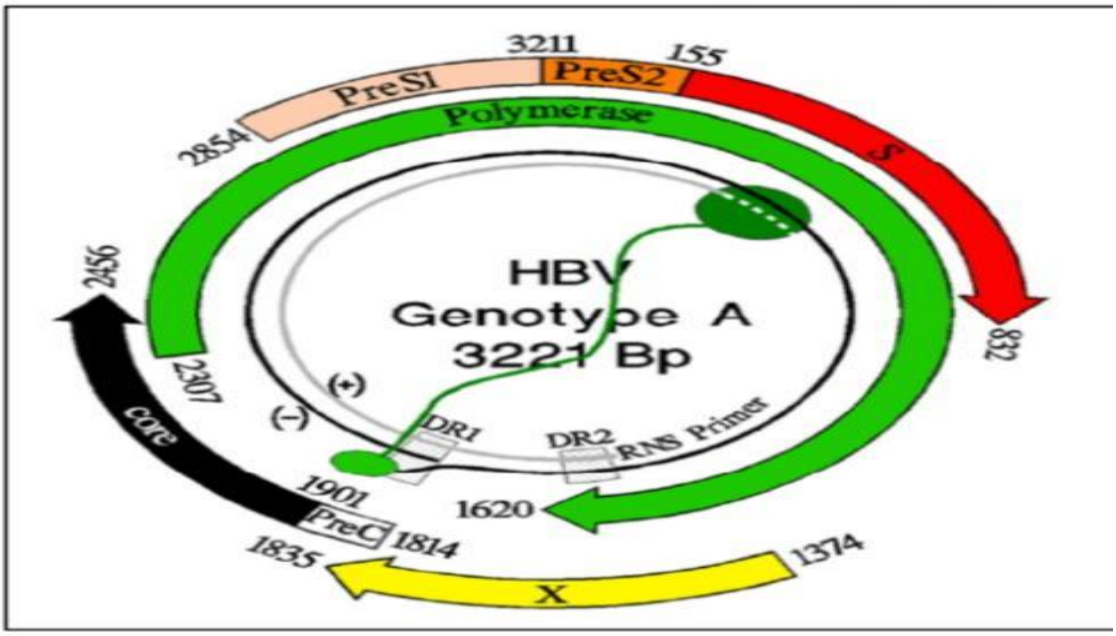


Fig 1.1. Hepatitis B virion structure shown schematically.

Transcription of proteins from the positive strand using covalently closed circular DNA (cccDNA). Seeger et al. (2007) and Bowyer and Sim's (2000) research are two examples of studies that are applicable to this discussion. The nucleocapsid surface protein is a lighter shade of blue, approaching turquoise, whereas the core protein is a deeper shade of blue. Different sized Perkins HBs were produced that year. When compared to DNA genomes, the rate of nucleotide exchange in viral genomes is 104 times greater, ranging from 0.1 to 0.7 per year (Bowyer & Sim, 2000; Zhu et al., 2010). RNA acts as a go-between in the replication process because the enzyme responsible for viral reproduction, reverse transcriptase, has a high mistake rate and poor proofreading skills.

In Figure 2, two enhancers and four promoters for viral RNA production are shown. Seeger et al. (2007) found that the length of pre-core/core mRNA was 3.5 kilobases, whereas the length of pre-S mRNA was 2.4 kilobases. S mRNA was measured to be 2.1 kilobases in length, whereas X mRNA was measured to be 0.7 kilobases.

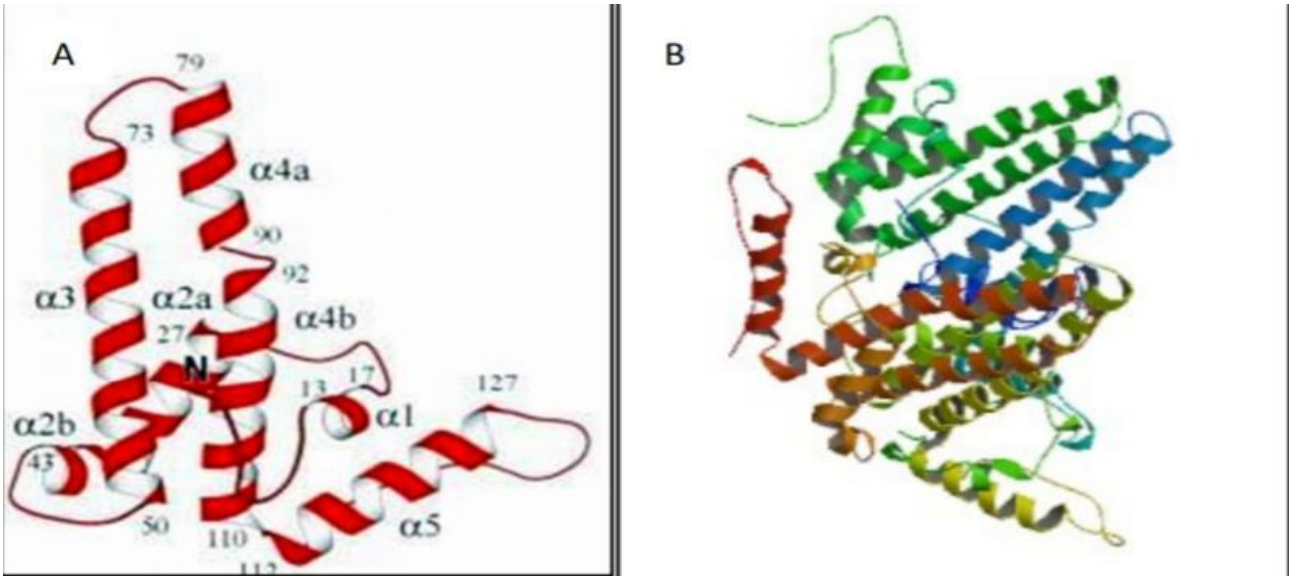
Primer design and genomic sequence interpretation are also affected by the sequencing strand, mostly because of differences in strain mapping. Valenzuela et al.'s stranded reference sequence, which they submitted using ECORI codes, is a match for genebank accession X02763.



The nucleotide values of the EcoR1 site for the proteins of the four overlapping reading frames inside the HBV genome are shown in Figure 1.2.

HBc

In question is a pure form of protein that hasn't been tampered with in any way. Since the virus was first discovered, researchers have used the virus's core protein's antigenicity to identify and track infections (Seeger et al., 2007). HBcAg's main antigen is made up of 183 different amino acids. The synthesis of dimeric capsid proteins requires the enzymatic degradation of the quantity of amino acids at N-terminal position 149. Each of the two parts of the HBa Ag dimer consists of four bundles of alpha helices. There are 90–120 triangular dimers in the capsid's quaternary structure, and four of these dimers come together to form a spike. Additionally, the dimers are covalently bonded to neighboring alpha-helices.



Monomeric HBV Core protein has a ribbon-like conformation (A) in its tertiary structure (Wynne et al., 1999). The core protein has been shown to be dimeric using X-ray crystallography and the Protein Data Bank entry 1QG.

HBE

The solvent protein pre C releases a smaller protein called e-antigen (HBeAg), which regulates the host immune response during HBV infection (Seeger et al., 2007). There are distinctions between HBcAg and HBeAg antigens (Watt et al., 2010). Antigenic specificity, solvency, features of data collecting, occupational exposure energy, and other examples fall within this category.DNA polymerase protein is abbreviated p-protein.The nucleotide sequence 2307-1620 provides the genetic coding for the pol quality of the viral DNA polymerase. Upstream reverse transcriptase, upstream RNAseH, and a DNA strand introduction for the less abundant strand are the three functional components of the item.

HBx

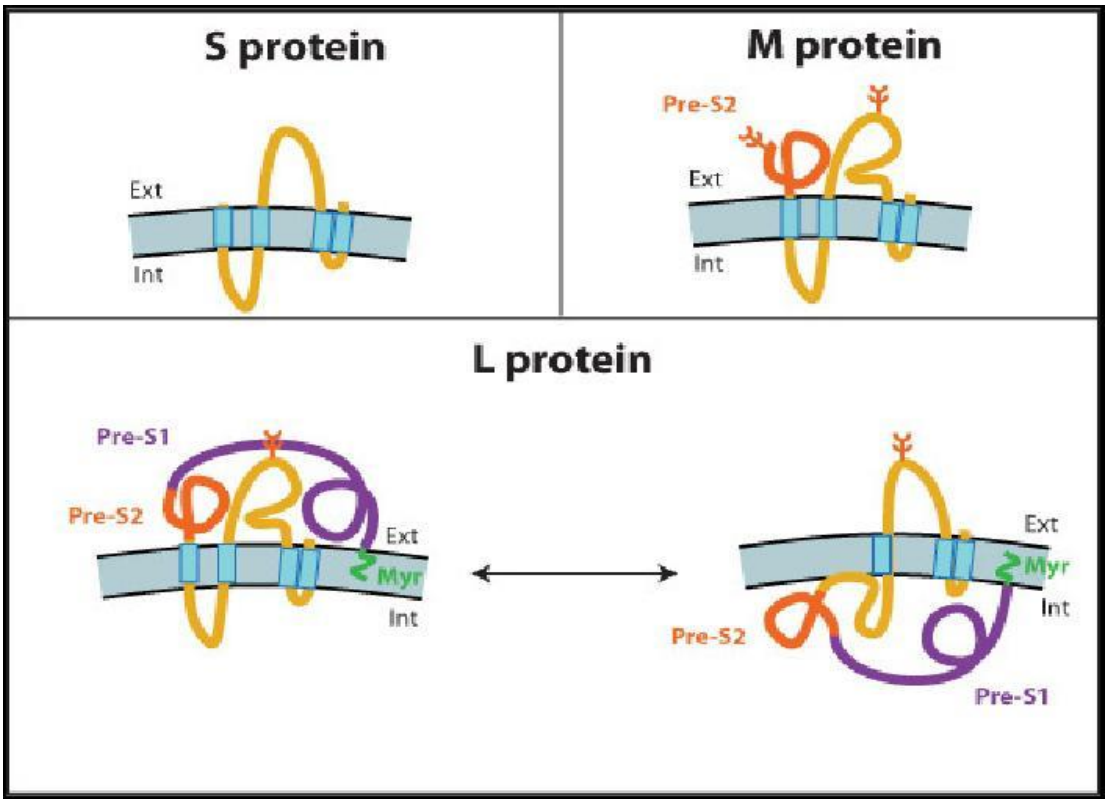
Hepatitis B X antigen (HBxAg), also called X protein, is located deep inside the virus's nucleus and is thought to have a connection to the cytoskeleton. This feature is sometimes seen in hepadnaviruses from animals. Toh et al. (2013) and Seeger et al. (2007) report that HBx interacts with NF-kB, AP 12, cEBP, ATFCREB, and NFAT limiting sites, all of which are cellular

transcription factors. The possibility for hepatocarcinogenesis has been linked to these relationships.

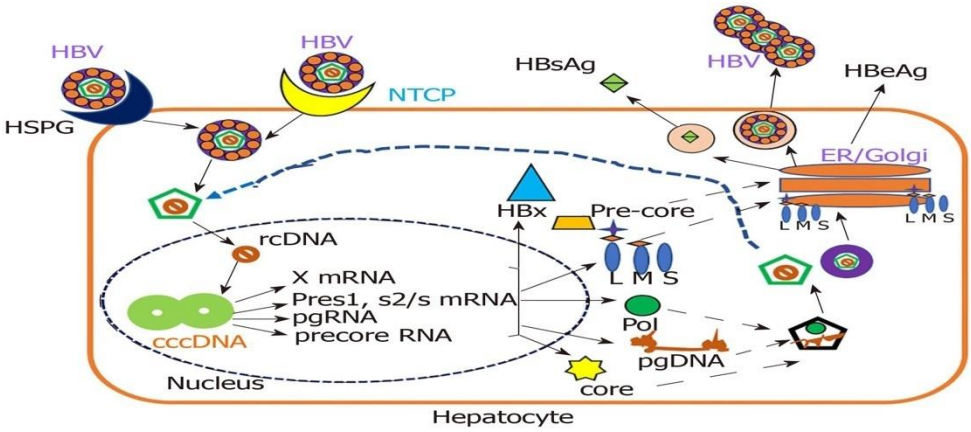
HBs

Pre-SS (2854-832) refers to the three transmembrane glycoproteins present in viral membranes. Proteins with myristolated L-forms are made by the S open reading frame, pre-S1, pre-S2, and S regions (PDB 1KCR). The protein specifically binds to viral receptors on hepatocytes. M-protein, whose translation begins at a start codon inside the protein's secondary structure, displays recognition of a structure larger than HBsAg but smaller than L-protein.

The first 55 amino acids of the M-protein are particularly reactive and interact with the pre-S2 region. Since the protein has no detectable effect on virion assembly, its exact functional capabilities are unknown. The smallest form of the S-protein, HBsAg, contains the crucial antigenic markers used to identify HBV. These markers are employed in the diagnosis of active infections and in the development of HBV vaccines (Seeger et al., 2007).



The following image depicts the two different L HB conformations described in the 2011 paper Viral Zone. Before DNA sequencing became commonplace, serologists used HBsAg to



categorize HBV strains based on large differences in the amino acid sequence of its structural components. ADW2, ADWRQ+, ADWRQ-, and ADWR are the most common serotypes. The predicted amino acid sequence (124-147) by Locarnini and Yuen (2010) places the start of the antigenic loops at positions 137 and 138. Locarnini and Yuen (2010) found that all serotypes of HBsAg have a common antigenic determinant that is exceptionally well preserved. The substitution of arginine for lysine at either position 122 or 160 in the protein is thought to be responsible for the occurrence of Yd and wr variants. The differences between w12 and w3, w4 and thr, and w4 and leu are encoded at position 127. Most strains express the q determinant, which is made up of amino acid residues 177 and 178 (Norder et al., 2004). In Figure 1.5, HBsAg may be observed.

Figure 1.5 is a schematic illustration of the principal antigenic determinant (a-determinant) of the HBV surface antigen and its accompanying major and minor loops. Mutations in the Overlapping polymerase gene have been linked to resistance to antiviral drugs, as seen by the darkened regions, as reported by Locarnini and Yuen (2010).

THE SPREADING AND MULTIPLYING PROCESSES

In the first stage, viruses bind to host cells. In a duck HBV model, we found that the N-terminal S-domain (amino acids 1-23) and the pre-S2 domain translocation motifs (TLM) peptide interact with carboxypeptidase D (CPD). Individuals are the focus of Schadler and Hildt's (2009) research.

Schadler and Hildt (2009) found that HBV has persisted in spreading while facing challenges such connection breakdown and cloaking. This discovery raises the possibility that HBV is spread either automatically or after an intentional period of covert exchange. Figure 1.6 from (Schuktz et al., 2004). Relaxed circular DNA from the genome is converted into complementary circular DNA (cccDNA) in step four, and this cccDNA then acts as a transcription template in step five. Seeger et al. (2007) and Schadler and Hildt (2009) conducted the research. Sub-genomic and pre-genomic RNA molecules are transported from the nucleus to the cytoplasm with the help of post-transcriptional regulatory mechanisms, as established by Seeger et al. (2007).

Four sub-genomic mRNA ORFs code for functional proteins: HBcAg, HBsAg, HBe Ag, HBx, and p. The signal is a stem-circular structure seen in pre-genomic RNA; it is called the kramvis and kew (2002) signal. It helps the chaperone and the p protein interact during the detection of stage 8 encapsidation sites. Protein dimers develop at the nucleation sites of pgRNA-p, which then polymerize to produce frame polymers. Seeger et al. (2007), Schultz et al. (2004), and Schadler and Hildt (2009) have all conducted considerable research on the embryonic RNA nucleocapsid.

A full nucleocapsid is formed once the e signal detects replication has begun and RNA begins the laborious process of transcribing record stage 9 into DNA in the cytoplasm. The endoplasmic reticulum, golgi complex, and pre-S and post-S sections of the large surface protein all play a role in preparing the nucleocapsid for transportation to the core during stage 10 (Schultz et al., 2004; Schadler and Hildt, 2009), which in turn allows the replication cycle to continue.

Hepatitis B virus (HBV) replication might be sped up by the presence of other disorders (Capobianchi, 2013; Bowyer & Sim, 2000; Simmods& Midgley, 2005). Beerenwinkel, Gunthard, et al. (2012) did research on the importance of protecting genetic diversity in highly fragmented communities at risk of contracting contagious illnesses. The research also investigates the factors that encourage the creation of viral quasispecies.

About 240 million people are chronically infected with hepatitis B virus (HBV) across the globe (Seeger et al., 2007; globe Health Organization, 2013), making it a major public health problem.

DIAGNOSIS USING LABORATORY TESTING

Testing for relevant antigen antibodies, such HBcAb, is a part of the diagnostic procedure. Core antibody subtyping has the potential to distinguish between acute (IgM class) and chronic (IgG class) HBV infection. The detection of hepatitis B virus (HBV) DNA is often employed as an additional indicator of the severity of HBV infection.

In order to determine the prevalence of infections between 1985 and 1992 for each of the eight (23) possible combinations (presence or absence) of the three primary serological markers, the South African National Institute of Virology (NIV), now known as the National Institute of Communicable Diseases (NICD), used data mining techniques. In order to automate the interpretation of laboratory results, the research team compiled a large database of diagnoses and accompanying notes (see table 1.1). Measurements of ALT and HBV DNA may be useful in establishing disease progression. Two of the eight possible combinations of tests (stage II and stage II) still lack enough data to be considered reliable, however.Over time, HBsAg titres decline.

Decreases in levels of pollution in the air. Hepatitis B surface antigen (HBsAg) levels dropping below the detection threshold, which might indicate the success of the treatment, are very unusual. The fact that HBV DNA may still be found in the circulation is, however, evidence that total eradication has not been accomplished. The medical community is at a loss to explain these illnesses. Liver tissue, serum, peripheral blood mononuclear cells, and other lymphoid tissues may harbor trace amounts of hepatitis B virus (HBV) DNA that can be detected with whole genome PCR amplification tests in cases of chronic infections (Gunther et al., 1995). The Pre-S1/S2 and S regions have been modified as a consequence of A-D recombination. Allain et al. (2009) found that among South African blood donors, the detection of HBsAg had a false negative rate of around 1 in 4067. In this discussion, we'll look into serology and how it applies to a wide range of diseases.

In order to make educated judgments and lessen the impact of any unfavorable results, it is crucial to weigh the risks associated with a certain activity or scenario.

The prevalence of viral hepatitis among medical professionals is high.Hepatitis virus infections are more common in certain population subsets and geographic areas. If a caregiver gets hepatitis through touching infected blood, they might potentially spread it to a patient.Users of intravenous drugs (IDUs) pose a threat to public health because they may transmit

communicable infections. Transfusions may expose this group to the danger of contracting viral hepatitis.Transmission of infectious diseases from mother to child may result from a lack of attention to hygienic procedures and a lack of medical understanding during labor and delivery.

The transmission of diseases inside families was also studied by the researchers. The spread of hepatitis might be facilitated by the lack of adequate hygienic procedures in barbershops. Blood donation and transfusion are the principal vectors for the spread of bloodborne diseases. It's crucial to remember that untested blood might transmit harmful blood cells. Human contact is a key factor in the spread of hepatitis viruses. The fast spread of the virus has been linked to high-risk sexual activity, such as having intercourse with several people. This is a common mode of transmission for viruses. Blood contamination and needle stick occurrences are much more common as a result of the rising number of people getting dental work done.

A complete analysis requires collecting and reviewing data over a certain time frame, which is referred to as the analysis period. It is aBilal Hospital's blood bank in Rawalpindi collected its data from the beginning of 2020 to the end of the following year, on October 31, 2021.

LEARNING ENVIRONMENTS

Bilal Hospital, the only publicly funded hospital in Pakistan with a blood bank, was the site of the study. The donation of blood is a typical way for people to help others in need. Most of the 35,000 people who get blood transfusions each year are men. In October of 2021, researchers analyzed a group of 966 blood donors for the presence of anti-HBc and other hepatitis B virus (HBV) antigens. Donations were not accepted from anyone who had recently been diagnosed with jaundice, had hemoglobin levels below 13 g/dL, had BMIs below 50, or who had fevers on the day of donation. The median prevalence of HBsAg, Hostile to HCV, anti-HIV, intestinal disease, and syphilis indicators in blood samples was determined by analyzing data from screenings performed between January 1, 2020, and October 31, 2021. Analytical sampling is the practice of selecting and collecting samples that are meant to be representative of a broader population for the purpose of analysis.

Each donor's blood was drawn using a syringe and needle measuring 5 cubic centimeters in total capacity. The blood samples were then centrifuged in an A vacutainer at a rate of 1500 revolutions per minute for 3 minutes to aid the separation of serum. The Guangzhou Wondfo

Biotech Co. is a Chinese biotech firm with headquarters in the city of Guangzhou. Ltd. The HBsAg test kit with the one-step cassette format accurately detected a concentration of less than 1 ng/ml. Discrete test strips were used to examine each serum sample. After 10 seconds, the strips were removed and dried for 15 minutes on a nonabsorbent surface before being analyzed.

Methods for Detecting Human Papillomavirus (HPV) Using PCR and Serology

Donor blood samples were tested for HBsAg, anti-HCV, and anti-HIV using commercial compound immunoassays (EIAs; AxSYM, Abbott Labs, Abbott Park, IL). Between January 2020 and October 2021, a total of 94,177 participants were surveyed. The Treponema pallidum hemagglutination assay (TPHA) from Crumlin, UK-based Randox was used to diagnose syphilis, while the presence of malaria was confirmed by analyzing thick blood films dyed with ethyl-Enediaminetetraacetate. Antibody testing for HBc (Center) (AxSYM, Abbott Labs) was performed in October 2021 on 966 blood donors. A secondary aggressive anti-HBc EIA (DiaSorin, Saluggia, Italy) was used to evaluate the reactions. Supplier-provided absorbance measurements were put to the test by choosing a value that was somewhat off from the calibrators' average. If the tests didn't achieve both goals, then they couldn't be trusted.

Commercial kits (AxSYM, Abbott Research Centers) were able to identify HBeAg, anti-HBe, and HBsAg in HBc-negative samples after they were stored in aliquots at 30 degrees Celsius. Polymerase chain reaction (PCR) was used to identify HBV DNA, and a commercially available kit (BioSewoom; Seoul, Korea) and piece of machinery (GeneAmp 5700; Applied Bio-Frameworks; Foster City, California) were used to do so. After amplification, the DNA sample was run through an agarose gel UV transilluminator (UVItec; Cambridge, UK) set to 150 V for analysis. Two independent checks confirmed the reliability of the HBV DNA results. The manufacturer predicts a detection limit of 50 DNA copies per milliliter in serum.

Authentication

AccuBioTech Co.'s products and services were utilized in this research. Rapid testing made possible via the use of multi-use cassette devices. Two drops of the sample and one drop of the kit were applied to the instrument for in vitro testing, and the instrument was incubated for five minutes before a result was recorded.

Establishing and using the same file formats, data structures, and definitions across an organization is what we call "data standardization."

SPSS, Adaptation 10.0 (SPSS Inc., Chicago, IL) was used on a personal computer to analyze the data. The chi-square test and Fisher's exact test were used to analyze data from frequency and rate surveys in this research. For a t-test comparison between anti-HBc positive and negative groups, the mean and standard deviation of blood donor time are provided. P values less than 0.05 were considered significant in this study.

In this research, we look at survey data and try to make sense of it.Over the last 15 to 20 years, the prevalence of HBsAg among healthy people in Pakistan has decreased significantly, from 813 to 10 to 15%. Rawalpindi and Islamabad have a high Hepatitis B Virus (HBV) prevalence, according to the World Health Organization (WHO). If hepatitis B were to be added to Pakistan's existing newborn vaccination schedule, coverage among infants would likely increase to 65.5% by 2019. The observed decline in hepatitis B virus (HBV) positive cases may be attributable to the improved reliability of HBsAg EIA testing.

Over the previous decade, the vaccination rate for healthcare workers against hepatitis B at Bilal Emergency Clinic has increased significantly, from 86% to 98% (unpublished perspective 16). However, several regional practices have not seen comparable changes (5). These include the reusing of disposable and glass needles, the use of potentially contaminated razors by hairstylists, and the prevalence of unlicensed dentistry clinics. The prevalence of Hepatitis C Virus (HCV) is predicted to remain unchanged at 2.4% in 2019 and 4.16% at now, despite the availability of effective antiviral medication. The prevalence of HBsAg positive among blood donors is comparable to that seen in India, falling somewhere between 1.7% and 2.2%. This framework has the potential to improve blood donors' knowledge of illness prevention, treatment, and prognosis. Twenty-two percent of the 966 people who tested positive for HBsAg also tested positive for the presence of a particular factor, according to this study. Additionally, the percentage of Indian replacement blood donors that tested negative for HCV was much lower, falling between 0.25 and 0.918% and 0.21 percent. In addition, just 0.50% of Indian donors tested HIV-negative.

About nine percent (9%) of the total sample size of 966 blood donors tested positive for Hepatitis B Virus (HBV) when tested for the presence of Hepatitis B core antigen (HBc). Nine

percent (9.6%) of the whole sample size showed immunity to HBc by testing negative for HBsAg; this included 167 people. The viral signature is independent of environmental conditions. Researchers found that 14.95 percent of Greek blood donors and 10.82 percent of Indian blood donors were HBsAg-negative and HBc-resistant. These results point to the growing adoption of routine disease screening and immunization regimens. Donors who test positive for HBc produce more anti-HCV antibodies than those who test negative for HBsAg. Anti-HBc sentiment is characteristic of the HCV transporter area. At the moment, Anti-HBc can only be used to detect people who are HBV carriers in their last stages of infection. However, a threefold increased risk of non-A, non-B hepatitis was seen among receivers of blood that tested positive for Anti-HBc compared to those who received blood that tested negative for Anti-HBc. Concerns have been raised about the quality of Pakistan's blood supply after HBV DNA was found in five serum samples that were negative for HBsAg but positive for Anti-HBc. as Shown. It is possible to classify the donors into three groups: those who are anti-HBc positive only (3), those who are anti-HBc positive in addition to being anti-HBe positive (2), indicating that they are in the recovery phase of HBV infection, as evidenced by the presence of anti-HBs and a decrease in HBsAg below detectable limits, and those who are chronic carriers with HBsAg below detectable limits (2). It is important to note that even if a patient's clinical state improves, leading to the disappearance of HBsAg, the generation of anti-HBs, and the restoration of normal liver function, there is still a possibility of detecting low quantities of HBV DNA in the bloodstream. This was observed in Pakistan, where 0.53% of blood donors who were found to be negative for HBsAg also tested positive for HBV DNA. There may be trace levels of HBV DNA in serum, and studies have shown that cytotoxic white blood cells may eliminate the virus without damaging hepatocytes. Transmission of HBV from donors who test negative for HBsAg is a real risk in Pakistani blood bonding facilities due to the absence of antiHBc screening. Transfusions acquired from donors that test positive for anti-Hepatitis B core antibody (antiHBc) are preferred by patients with compromised immune systems because they are devoid of Hepatitis B Virus (HBV) DNA. Blood donors who tested positive for HBs antigen exhibited an immunological response of more than 100 mIU/mL in 87 (67%) of the cases. In contrast, more than 30 mIU of neutralizers were present in 86% of those who tested negative for HBs antigen.

First-time contributor involvement would reduce by 17% if ideas inspired by HBc were disregarded. Most Pakistani bonding facilities lack the resources to do HBV DNA screening on

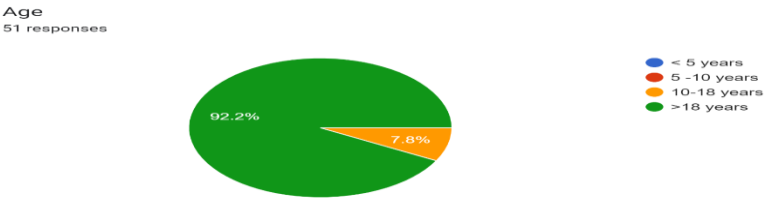
all HBc-positive blood donations, despite the obvious advantages of doing so. Due to their positive status for different illnesses such HBsAg, HCV, HIV, TPHA, and malaria antigen, around 7% of potential donors are excluded from participation.

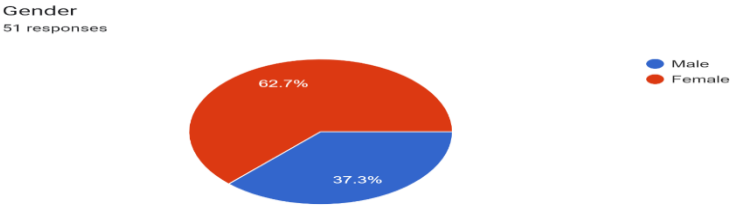
Many blood banks lack both trained staff and enough funding to purchase necessary equipment. In the context of the transfusion-based system, it is crucial to stress that screening donors for HBc, with or without HBV DNA, remains a difficulty even in rich socioeconomic situations or well-equipped blood donation facilities. Nucleic acid testing is challenging with current techniques since most blood donation facilities cannot screen for EIA viruses. Proactive recruiting of volunteer blood donors by transfusion centers may improve the efficacy and cost-efficiency of Anti-HBc screening. Blood banks need to aggressively seek for a large pool of donors who routinely test negative for HBsAg and anti-HBc in order to keep up with the demand. These givers need encouragement and help. This group will be tested for HBsAg, anti-HCV, and anti-HIV during future visits to the blood transfusion facility. Blood transfusion clinics should emphasize selecting healthy young donors aged 18-25 due to the reduced risk of Hepatitis B virus (HBV) transmission due to the existence of antibodies against Hepatitis B core antigen (HBc) in traditional screening procedures. The people who are immune to HBc and who test negative for HBsAg should be drawn to the bonding facilities. Encourage regular blood donations within this population. It is advised that these donors be tested for HBsAg, HCV, and HIV at the blood transfusion clinic. People between the ages of 18 and 25 have a lower chance of getting Hepatitis B Virus (HBV), making them an ideal population to target for scheduled blood drives. Therefore, due to limited resources, anti-HBc testing may be limited to first-time blood donors exclusively. Those with anti-HBs levels of 100 mIU per mL or more and a negative HBV DNA test result are considered suitable blood donors. This method may allow for more efficient use of funding provided by benefactors. After updating its federal and provincial blood transfusion systems, Pakistan may decide to adopt this strategy. Volunteer blood donor recruitment is a problem for many hospitals, as is keeping blood and blood products in stock. This means that people all around the country have access to these centers' full blood transfusion services. In its current form, the evaluation ignores regional differences in prevalence rates. As a result, a major effort from the public or private sector, together with substantial investments in infrastructure, trained employees, and equipment, are required to develop voluntary blood

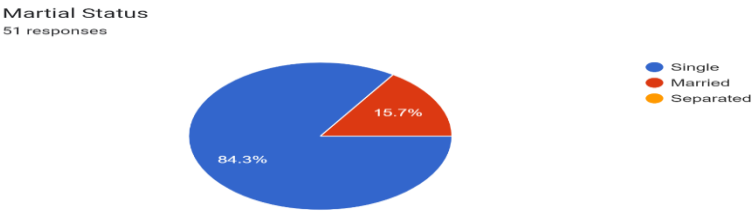
donation programs and improve blood safety. Therefore, there is a chance that prevalence studies are inaccurate.

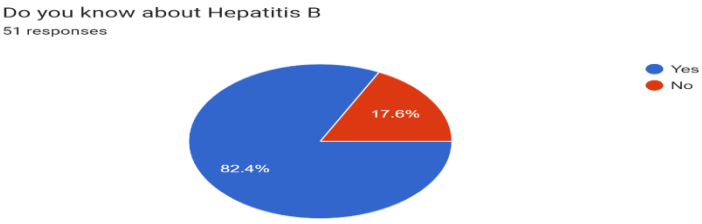
RESULTS AND DISCUSSION

Questionnaires Response

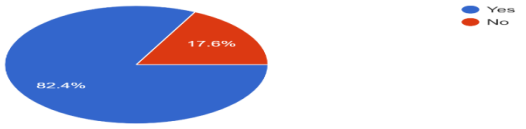




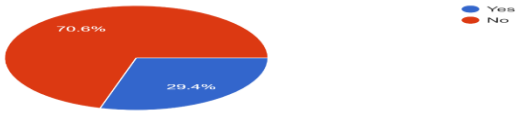




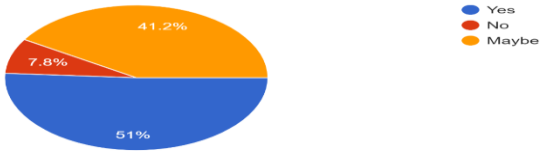
Do you know about Hepatitis B
51 responses



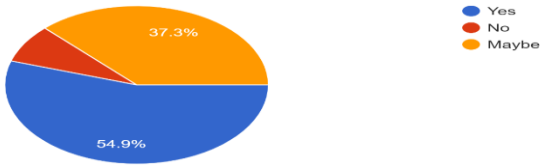
Do you have family history of Hepatitis B
51 responses



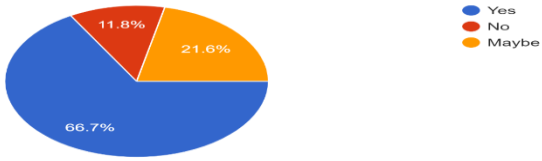
Hepatitis B can be transmitted through sexual contact
51 responses



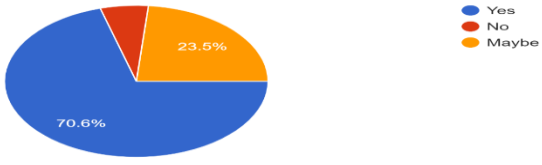
Hepatitis B can be transmitted from mother to child at birth
51 responses

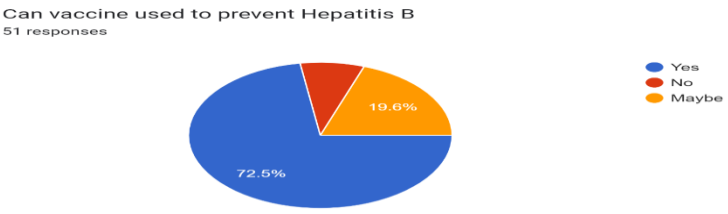


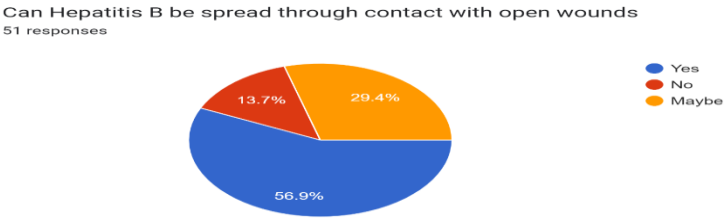
Hepatitis B can cause liver disease
51 responses

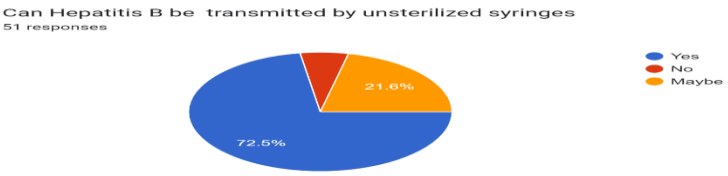


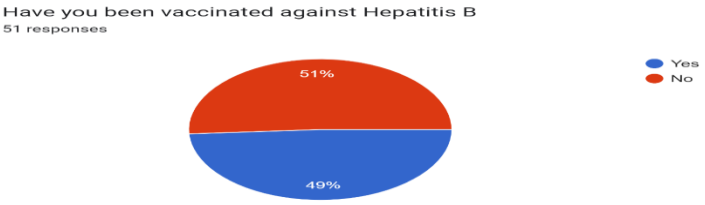
Hepatitis B can be cured?
51 responses

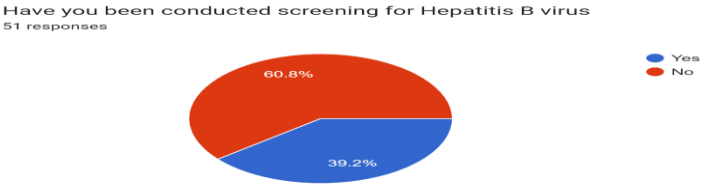












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ANALYSIS OF VARIOUS LIGNOCELLULOSIC SAMPLES FOR PR ODUCTION ALCOHOLIC FUEL

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1. **Must , Mirpur AJK**
2. **Grand Asian University Sialkot**
3. **UIBB-Arid Agriculture Rawalpindi**

ABSTRAT

A approach for the management of agriculture as well as other organic wastes utilization for production of alcoholic fuels like bioethanol and biobutanol. Therefore cellulosic materials like wheat , cotton and rice straws, corn stover , fruit wastes and cogon grass were used in this study Biological and chemical pretreatments were compared for each substrates. Efficiency of bacterial enzymes for saccharification of agricultural substrates was evaluated. It is concluded that these bacterial enzymes have the potential to hydrolyze not only pure substrates but can also degrade agricultural wastes. It is expected that outcome of this study will help to increase production of biofuels and to reduce burden of foreigner exchange that is currently being utilize to import fossil fuel from other countries.

Key words; Alcoholic fuel Green house gases, Climatic changes

INTRODUCTION

Exploration of sources for alternate energy have been increasedbecause of increasing concerns about energy security and climate change. The transportation sector plays a significant role for emission of greenhouse gases due to uses of fossil fuels, However, replacement of oil derived fuels such as ethanol could reduce environmental impacts and give advantages on social as well as economical levels (Humbird et al,2011). Various alternatives to generate sustainable biofuels are being investigated. Biological energy resources arelike bioelectricity, biogases, biodiesel and bioalcohols. Among these sources, bioalcohol shows a great potential to reduce the emission of greenhouse gases, decrease the dependence on fossil fuel and act as a chemical feedstock and fuel for transport (Dhamole et al., 2015).The production of bioethanol has been improved

extremely because many countries are trying to reduce the import of oil, improving the quality of air and growing rural economics. The global ethanol production is 51,000 million liters(Renewable Fuels Association, 2007). Ethyl alcohol has some advantages as a fuel as it has higher oxygen contents. The higher oxygen level permits improved oxidation of hydrocarbons with successive reduction in aromatic compounds and carbon monoxide emission. Ethanol has greater octane rating properties (Thomas and Kwong, 2001).

Biomass is a vital energy resource in Pakistan because ofagricultural based country. The biomass produced in livestock and agriculture sector in the form of animal waste and crop remaining as sugarcane bagasse and rice husk (Amiri et al. 2014; Chaudhry *et al*., 2009). Second generation biomass is mainly composed of lignocellulosic material. Lignocellulosic biomass is more plentiful organic substance on earth and consists of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%)(Huber et al., 2006).Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds (Demirbas, 2001). Most commonly used feedstocks are wheat straw, wheat bran, corn stover, corn steep liquor and apple pomace (Ejezi*et al*., 2006).Now a day, agricultural waste is used for the production of biofuels like biodiesel, bioethanol, biohydrogen and methane as compared to energy crops because they have competition with food crops. As huge amount of agrowaste is available and have discarding problem so, alternate option is the utilization of lignocellulosic biomass in order to reduce the competition between fuel and food (Mahro and Timm, 2007). The grasses are considered as reliable substance for extraction of ethanol. The utilization of perennial grasses is advantageous and possibly it further decreases the cost for the production of ethanol and its use as fuel (Gomez et al., 2008).

The Cogon grass (*Imperatacylindrica*) can be grown all over the yearworldwide, particularly in subtropical as well as tropical countries. Cogon grass has been exploited to rise the soil stability and as fodder, it is recognized as worst weed and it is known as pest by almost 73 countries in all over 35 crops. The roots of cogon grass have secondary metabolites which have medical importance. It is known as perennial grass and could be cultivated in any soil which usually considered as unfit for production of crops. The cogon grass could be utilized as a raw material for renewable source of energy (Lin and Lee, 2011).The *Cynodondactylon* (Coastal Bermuda grass) is perennial grass that has the higher cellulosic content and can also be used for ethanol production. The excellent raw material for yield of ethanol is coastal Bermuda grass as it is either sold at a very cheap price or is wasted in most of the cases. Comparison of corn and Bermuda grass has shown that most potential source for production of ethanol is Bermuda grass because of higher contents of biomass and conversion of whole carbohydrates into bioethanol. Bermuda grass is predominantly present in tropical and subtropical part of the world. It reaches 1-30 cm in height and have deep roots up to 2m into the ground, however various roots penetrated less than 60 cm under-ground (Sun and Cheng, 2005).Even though some species of Bermuda grass can grow up to 15-20 cm, others may reach a height of greater than 1m long. Bermuda grass can naturally grow in many continents such as North Africa, southern Europe, Asia and Australia (Sluiteret al., 2008).

In the present study various agrowaste samples such as wheat, cotton and rice straws as well as corn stover, cogon grass and peels of fruit wastes were selected as these are main organic wastes in Pakistan. Other than wheat and rice the third vital cereal is corn. Its production is 4.695 million tons annually and grown in 1130 thousand hector. The second major crop is cotton and it is cultivated annually. The third important crop is rice and it is cultivated at 2891 thousand hector and it is produced 7005 thousand tons annually (PES, 2014-15). Various studies reported that 2.7 million tons waste such as rice straw, rice husk, canola straw, wheat straw, cotton stalks, cotton bagasse and sugarcane remains are cultivated in Sanghar which is known as agricultural area in Sindh region of Pakistan. Almost 75-85 percent of these feedstocks are not utilized are burnt away. So, these materials could be used for production of energy and having no effect on food or other domestic resources (UNEP, 2011).Maize (*Zea mays*) is the best of the cereal stovers and very abundant livestock feed. It can be grazed off otherwise, it is mostly burned in fields in many areas of Pakistan before next crop to sow and all parts of maize are usable for different purposes (Kim and Dale, 2004). Termite gut has one of the highest microbe densities on earth. Termites depend on the microbes in their gut or digestive tract to digest the complex sugars in wood into simpler molecules . Cellulose is a major sugar in wood , it is broken down by bacteria available in gut of termite and finally converted into various products including fatty acids and alcohol like ethanol etc.(Kim and Haltzapple.2005).*Clostridium* **,**genus of rod shaped gram positive bacteria member of which found in soil, water and intestinal tract. *Clostridium acetobutylicum* ferments sugar to a mixture of organic solvents like acetone , butanol and ethanol.***Saccharomyces cerevisiae*** (known as baker s years) single celled eukaryotes which is frequently used in fermentation process for production ethanol and other alcoholic products.

MATERIAL AND METHODS

Collection of Samples

Various samples of wheat and rice straws as well as, peel wastes were collected from various areas . About 1 Kg samples of each samples were collected in fine plastic bags. The samples were shad followed by sun and oven dried for overnight at 55 °C. The samples were converted into fine powder form by electric grinder and passed through 40 mesh standard size

sieve. The powder form of samples were saved in fine plastic bags duly labeled with the name and were stored in refrigerator at 4°C till further uses.

Analysis of Samples

All samples were analyzed for ash contents, volatile matter, crude protein, crude fiber, crude fat and wet as well as dry weight (AOAC,1990). The standard methods were used for the estimation of total solids and moisture contents by drying at 105 ºC to remove moisture from the samples (Sluiter, 2005).

The cellulose content of sample was estimated by using reported method. The hemicellulose was determined by computing ADF (Acid Detergent Fiber) and NDF (Neutral Detergent Fiber) differences .The lignin contents were determined by standard method as reported by AOAC (1990).

Chemical Pretreatment

For chemical pretreatment two chemicals were used such as acid (H_2SO_4) and alkali (NaOH). Pretreatment experiment was performed by using H_2SO_4 and NaOH (1.0, 1.5 and 2%) at diverse temperatures such as 100 °C , 110 °C and 120 °C for different times durations (15, 30, and 45 minutes). Solid sample (10 %) (w/v) in reagent bottle was utilized during experiment. After pretreatment, the vacuum filtration assembly was used for filtration of sample in each bottle and the contents were emptied on filter paper. After filtration, the solid washed away with 300 ml distilled water in order to neutralize the pH. The filter paper was than dried at 105 °C and weighed.

Enzymatic Hydrolysis

The biomass after pretreatment 5% (w/v) was hydrolyzed with cellulose and β-glucosidases at 50 °C and 160 rpm for 72 hours in a water bath shaker with 0.05 M buffer (sodium citrate) at 4.8 pH. Cellulases having activity of (30FPU g-1). The samples were withdrawn from reagent bottle after every 12 hours to determine the concentration of sugar. After enzymatic hydrolysis, H_2SO_4 (µl) was added. Un-hydrolyzed sample was separated by centrifuging for 10 minutes at 13,500g. Supernatant was collected by means of syringe filters for sugar analysis by dinitrosalicylic acid

(DNS) method .The amount of sugar was analyzed by p-hydroxybenzoic acid hydrazide (PAHBAH) method. By using the concentration 1Mm-25mM of xylose the standard curve was drawn. Then by comparing the standard sugar concentration, the amount of sugar in pretreated sample was determined. The best pretreatment condition was selected after enzymatic hydrolysis process. The sample containing higher amount of released sugar was further selected for fermentation process. The solid biomass was stored at 4 °C which was then used for fermentation process .

Saccharomyces cerevisiae strain was maintained on YPD (yeast extract 1% (w/v), peptone 2% (w/v) and glucose 2% (w/v)] agar medium at 4°C. Culturing of yeast cells was carried out in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30°C for 16 h on a rotary shaker (100 r.p.m.) according to (Alfenoro, 2002)

Separate Hydrolysis and Fermentation

Fermentation experiment was carried out by using *C. thermocellum*grown in glucose yeast extract broth medium for 48 hours and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature (Jiang et al., 2015).Fermentation experiment was performed at 50^0C and 120 rpm for 72 hours under anaerobic conditions. After completion of fermentation reaction, the obtained mixture contains methanol, butanol, ethanol and acetone were removed by fractional distillation process in a fractional distillation apparatus on the basis of boiling point. As butanol has higher boiling point (118 ^{0}C) than water (100 ^{0}C) Butanol can be condensed then separated. The boiling point of ethanol is lower (78.3 ^{0}C) in comparison with water that's why it can be condensed earlier than water (Kathleem et al., 2018).

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Statistical analysis

Data generated through various analysis were statically analyzed for mean, standard deviation etc.,

Results and Discussion

Results regarding isolation of bacteria , chemical analysis of biomass samples as well as fermentation of sugars into acetone- butanol- ethanol are given in the following sections.Termites are considered as good source of various useful bacteria isolates those have industrial applications . These isolates are found to have good potential for conversion of various sugars into alcoholic products . Therefore in current study acetone- butanol - ethanol (ABE) were produced from organic wastes material of agriculture and municipal sources by using termite based bacterial isolates (Figures 1-2).

Biological Pretreatment

Results displayed in table 3 indicates amount of sugar released by different bacteria isolates. It was observed that bacterial isolates 9x (xylanase enzyme)has provided higher amount of sugar (27.84$\pm$0.48mM/l) from wheat straw (Table 3), which was higher than all other substrates analyzed.

Table1.Chemical pretreatment of biomass samples with different concentrations (%) of NaOH and H_2SO_4,to release of sugars (%).

Substrates	Chemicals					
	H_2SO_4 concentration			NaOH concentration		
	1%	2%	3%	1%	2%	3%
Wheat straw	15.38±1.24	19.74±1.25	6.38±0.86	14.71 ±0.46	15.95±0.08	16.85±0.15
Corn stover	14.57±0.18	13.73±1.12	4.27±0.81	13.69±0.46	13.65±0.08	13.82±0.14
Cotton stalk	1.53±0.04	1.35±0.05	0.28±0.02	0.86±0.06	0.87±0.11	0.97 ±0.01
Rice straw	16.85±0.15	15.38±0.17	3.44±0.15	15.07±0.17	14.32±0.28	13.39±0.59

Pretreatment of biomass samples

Table 2. Analysis of various products

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Bacterial Isolates	Corn stover				Wheat straw			
	Acetate	Formate	Lactate	Ethanol	Acetate	Formate	Lactate	Ethanol
Isolate 9x	1.15±0.06	_	1.41±0.18	5.73±0.28	3.04±0.65	_	1.65±0.79	3.34±0.41
Isolate 10	1.28±0.14	_	3.44±0.34	6.98±0.58	1.55±0.28	1.24±0.17	6.14±0.55	5.99±0.26
Isolate 31	1.29±0.34	1.98±0.39	8.57±0.59	9.21±0.54	1.72±0.07	1.63±0.28	3.58 ±0.26	6.43±0.49

Simultaneous Sccharification and Fermentation

It was observed that there are variation for growth of different isolates on differentsubstrates that might be due to availability of amount sugars and other similar byproducts

Data in table 3 represents various parameter found in biomass samples. Whereas ligno-cellulosic contents of the samples are given in table 4. It was observed that Cogon grass has higher cellulosic contents as compared to other substrates used for analysis.

Table 3; Proximate analysis (%) of biomass samples

Substrate	Dry Matter	Moisture	Crude Protein	Crude Ash	Crude Fiber

Peel(wastes)	92.41±0.48	7.53±0.34	7.93 ±0.23	5.91± 0.45	33.87±0.33
Cogon grass	93.11±0.27	6.89±0.26	5.12 ±0.21	9.18±0.34	35.41±0.42

Analysis of organic wastes samples

Table 4. Chemical analysis (%)of biomass samples

Substrate	**NDF**	**ADF**	**Hemicellulose**	**Cellulose**	**Lignin**
Peel(Fruit wastes)	79.6±0.51	52.1±0.31	26.3±0.34	29.6±0.67	21.5±0.43
Cogon grass	82.06±0.72	48.41±0.42	29.6±0.52	34.2±0.83	15.32±0.25

Mean ± standard deviation (n=3) NDF = Neutral Detergent Fiber and ADF=Acid Detergent Fiber

Table 5; Recovery of solid mass (%) due to treatment with Acid under various conditions.

Pretreatment conditions.		**Total solid recovery (g/100g dry biomass)**	
Time (min.)	**H_2SO_4Concentration**	**Peel (wastes)**	**Cogon grass**
15	1.0	63.52±0.20	76.86±0.61
	1.5	62.02±0.13	74.24±0.43
	2.0	57.11±0.34	70.33±0.25
30	1.0	56.61±0.43	68.86±0.48
	1.5	56.07±0.22	67.10±0.35

	2.0	52.37±0.20	64.76±0.24
45	1.0	53.07±0.32	64.62±0.42
	1.5	52.11±0.51	64.02±0.36
	2.0	51.23±0.44	63.50±0.56

Dilute H_2SO_4 pretreatment

The samples of the various biomass were pretreated with dilute acid 1, 1.5 and 2% concentration, an autoclave at temperature of 105, 120 and 135ºC for the period of 15, 30 and 45 minutes. The temperature 120 °C is considered best for both the samples while the retention time of 15 minutes was suitable for peel wastes and 30 minutes for cogon grass at the concentration of 1.5% and 1% respectively, these are the optimized conditions that was used for enzymatic experiment (Figures 6-7).

Fermentation

Cogon grass produces 10% of ethanol where as Peel wastes produces 7.4% of ethanol.as shown in Table 9. As the time period increases, glucose concentration was reduced but ethanol concentration was enhanced but up to certain time limit. However, after 72 hours glucose concentration was not sufficient to maintain the ethanol production. Higher cellulosic but lower lignin contents of cogon grass was compared to Peel wastes and it was found that these contents make cogon grass a better candidate for ethanol production.

Table 6. Ethanol production from Cogon grass and peel wastes samples

Sample	**Ethanol Production (% v/w)**
Cogon Grass	10.5

Peel wastes	7.4

Table 7; Sugars and other products (%) obtained from grasses

Substrate	Glucose	Xylose	Lignin	Dry matter	Moisture	Ash
Cogon grass	32.36. ±1.14	18.85 ±1.18	6.93 ±0.44	90.33 ±1.85	9.67 ±0.54	5.77 ±0.46
Peal wastes	27.32. ±2.15	15.37 ±1.13	4.75 ±0.54	92.46 ±1.24	8.56 ±0.55	4.89 ±0.58

% age values of various parameters of biomass samples.

Dilute sulfuric acid pretreatment of Peel wastes

Glucose content in peel waste was 27.33±2.15% (Table 10). There was increased in the glucose content after treatment (Figures 10 -11) The solid fraction of samples has given larger quantity of glucose when it was treated with dilute acid concentration (1.8 %) for 30 minutes and the temperature of reaction was maintained at 110 °C. It proved that moderate temperature and acid concentration play key role to enhance the glucose contents during pretreatment. Similar finding on acid hydrolysis of orange peel at low temperature has been reported by Talo et al. (2014).

Percentage decrease in xylose content in solid fraction of peel wastes after H_2SO_4Pretreatment

In peel wastes xylose content was 15.37%±1.13 (Table 10) and after pretreatment decrease in xylose content was observed. An acid concentration of 1.10% and incubation temperature of 110 °C for 30 minutes was found optimum to achieve minimum xylose in the solid fraction (Figure 11).With dilute acid pretreatment 100% removal of hemicellulose is possible (Sun and Cheng, 2005). Wyman et al. (2005) has also reported that maximum xylose solublization occurs at moderate temperature.

Percentage increase in lignin content in solid fraction after H_2SO_4pretreatment

Results indicates that optimum acid dose of 2.0% for 37 minutes at 125 °C is enough to get minimum lignin in solid fraction. Although the reaction has shown apparently 7.32% increases in lignin content but values in real sense was in decreasing order . The apparent increase in lignin content was due to huge removal of xylose after H_2SO_4 pretreatment. If the temperature is kept constant, then the solublization of lignin becomes higher with elevating reaction time to ascertain maximum value.

Table 8; Maximum Sugar yields after enzymatic hydrolysis of substrates at pH 4.8, 50 °C, 120 rpm.

Substrate	$Glucose_{SF}$ (2.5g/50mL)	$Glucose_{SF}$ (50g/L)	Glucose(g/L)	$Rate_{sac}$ (%)	$Time_{OPT}$(hours)
Cogon grass	1.10	22.00	17.72	80.54	72
Peal wastes	0.98	18.5	14.7	78.35	72

* SF = Solid fraction Y = Yield Sac = saccharification Opt = optimum

The reason behind higher saccharification (80.54%) was achieved as there was no accumulation of sugar like cellobiose occurred although cellobiose was available in reaction mixture. Xue et al. (2012) has also pointed out that the performance of celllases was actually

enhanced (due to absence of cellubioses), and the results in higher sugar recovery after enzymatic hydrolysis.

Table 9 .Chemical analysis of various crops samples

Parameters	**Cotton stalks**	**Corn stove**
Moisture contents	6.5	7.0
Volatile Matter	77.0	75.0
Fixed Carbon	9.5	19.5
Ash contents	8.7	6.0
Crude Fiber	31.0	32.0
Ether extract	1.8	2.5
Crude Protein	4.2	3.8
Cellulose	34.5	33.6
Hemicellulose	29.5	32.5
Lignin	14.8	18.5

Various parameters of bioma

Analysis of Sugar after Pretreatment and Enzymatic Hydrolysis

In this study three different substrates i.e. corn stover, wheat straw and rice straw were used for sugar production by enzymatic hydrolysis in 500 mL Erlenmyer flask at 50 °C for three days. For sugar production, pretreatment process of lignocellulose is necessary to break down lignin and it increases the accessibility of enzymes and microbes to carbohydrates(Figures 12-13). Two types of pretreatment methods have been applied on these substrates. In physical pretreatment these substrates were first groundto fine powder and passed through 80 mesh size sieve to reduce the size of particles. Then these substrates were subjected to chemical

pretreatment. In order to interrupt the structure of lignocellulosic biomass, acidic and basic pretreatment (Chemical pretreatment) conditions were applied (Table 6-9).

Dilute Acid Pretreatment

Different concentrations of sulphuric acid H_2SO_4 were used for pretreatment of agricultural waste samples. The samples were pretreated at a solid loading of 20% (w/v) slurry and heated at a temperature of 100, 110 and 120 ^{0}C in an autoclave. The reaction was performed at a retention time of 10, 15 and 20 minutes and at three different concentrations of sulphuric acid i.e. 0.5, 1 and 1.5%. At each temperature, substrate was pretreated with three different concentrations of sulphuric acid. A sample was pretreatedtriplicate at same temperature and reaction time. Total 9 treatments of three samples were pretreated at a time in 100ml reagent bottles. Total 9 experiments were done (9 × 9 = 81) so, 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for acidic pretreatment (Figure 12).

Dilute Alkali Pretreatment

Dilute alkali was used for pretreatment of agrowaste samples. Different concentrations of sodium hydroxide (NaOH) were used at different temperature and different retention time to optimize the condition which may give maximum yield of glucose. The samples were pretreated at a solid loading of 20% w/v slurry and heated at 100, 110 and 120 ^{0}C in autoclave for 10, 15 and 20 minutes of reaction time. For pretreating the sample ofdifferent concentrations of sodium hydroxide 0.5%, 1% and 1.5% were used. Total 9 experiments were done (9 × 9 = 81) , therefore total 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for basic pretreatment.

Spectrophotometric analysis and Comparison of sugar production in three agrowaste samples

Better glucose yields were obtained from wheat straw in all 9 experiments after 72 hours of enzymatic hydrolysis. It was observed during experiment that by increasing the concentration of H_2SO_4 from 0.5 to 1.5% the amount of sugar was also increased. In all experiments, higher yield of glucose was also recorded at a retention time of 20 minutes rather than 10 and 15 minutes. For acidic pretreatment conditions of wheat straw, the conditions were

optimized at 120 ^{0}C, 20 minutes of retention time with 1.5% of sulphuric acid (Figure 14). At this concentration, glucose concentration was at peak. During alkali pretreatment conditions, the glucose yield was increased by increasing the temperature and higher yield was recorded at 120 ^{0}C. Meanwhile at similar temperature high yield of glucose was recorded when 1.5 % concentration of NaOH was used. By increasing the time of enzymatic hydrolysis from 0 to 48 hours sugar yield was increased but when the time is increased further to 72 hours sugar concentration was chopped.The decrease in glucose concentration was probably due to production ofinhibitors by higher acid concentrations. Maximum reducing sugars (7.73 g/L) were obtained at 120^{0}C , when 1% NaOH concentration and reaction time of 15 minutes were used .

Rice straw has shown higher glucose yieldin acidic pretreatment conditions at 110 ^{0}C, acid concentration (1.5%) and retention time 10 minutes was used (Figure 15).The optimum condition used for rice straw analysis in case of alkaline pretreatment, temperature (100 ^{0}C), sodium hydroxide concentration (0.5%) and retention time (20 minutes) . Higher yield was obtained after 72hours of enzymatic hydrolysis (Figure 17).

During acidic pretreatment high yield of glucose was obtained at a temperature(120^{0}C), H_2SO_4 concentration (1.5%)and reaction time of 15 minutes (Figures 13-14). The optimum condition for corn stover at alkaline pretreatment condition was temperature 100 ^{0}C, concentration of sodium hydroxide (1.5%) and retention time applied was 20 minutes (Figure 18).

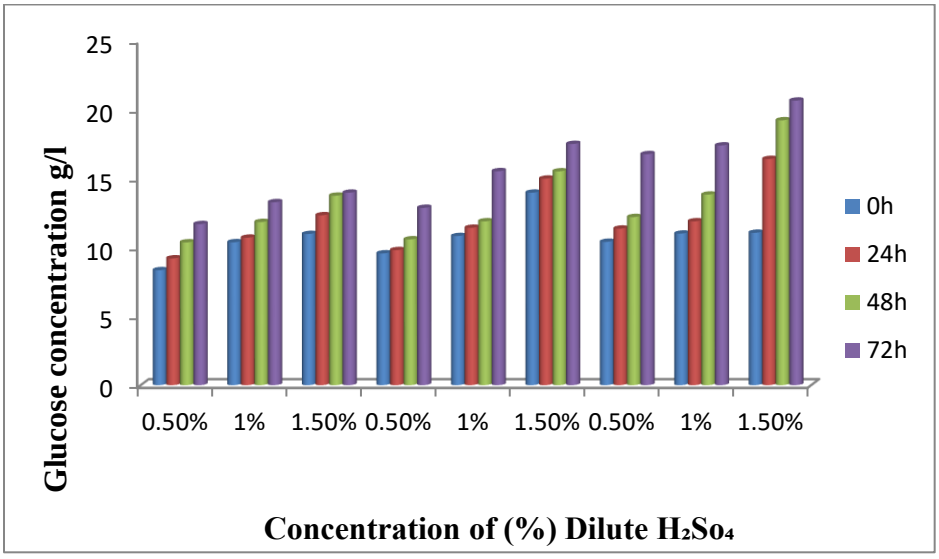

Figure 1. Glucose yield obtained from Wheat straw at H_2SO_4 pretreatment conditions at 120^{0}C

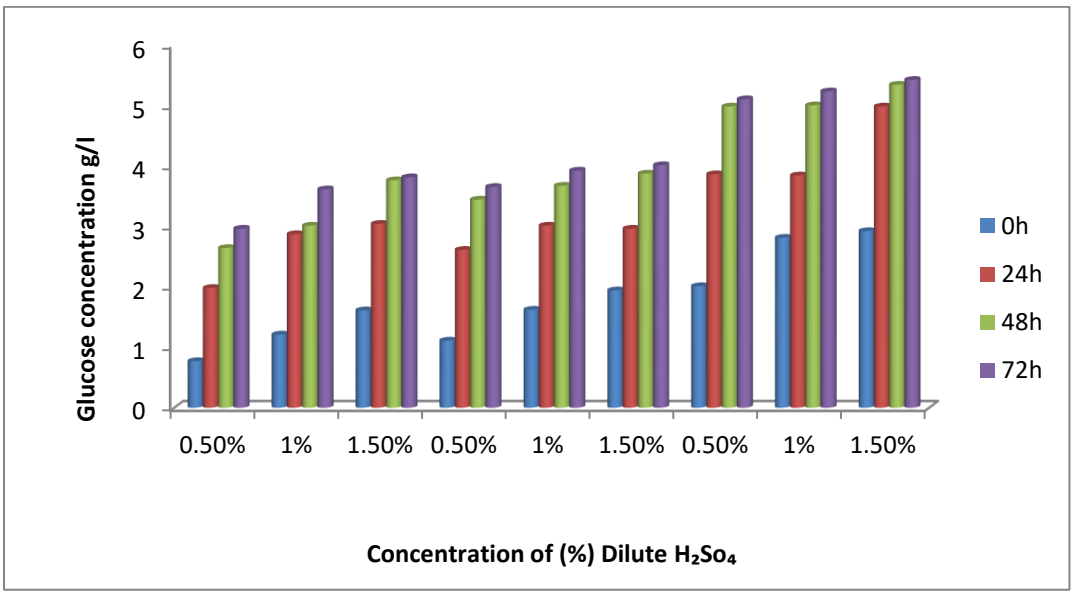

Figure 2. High glucose yield obtained from Rice straw at H_2SO_4 pretreatment conditions at 110 ^{0}C

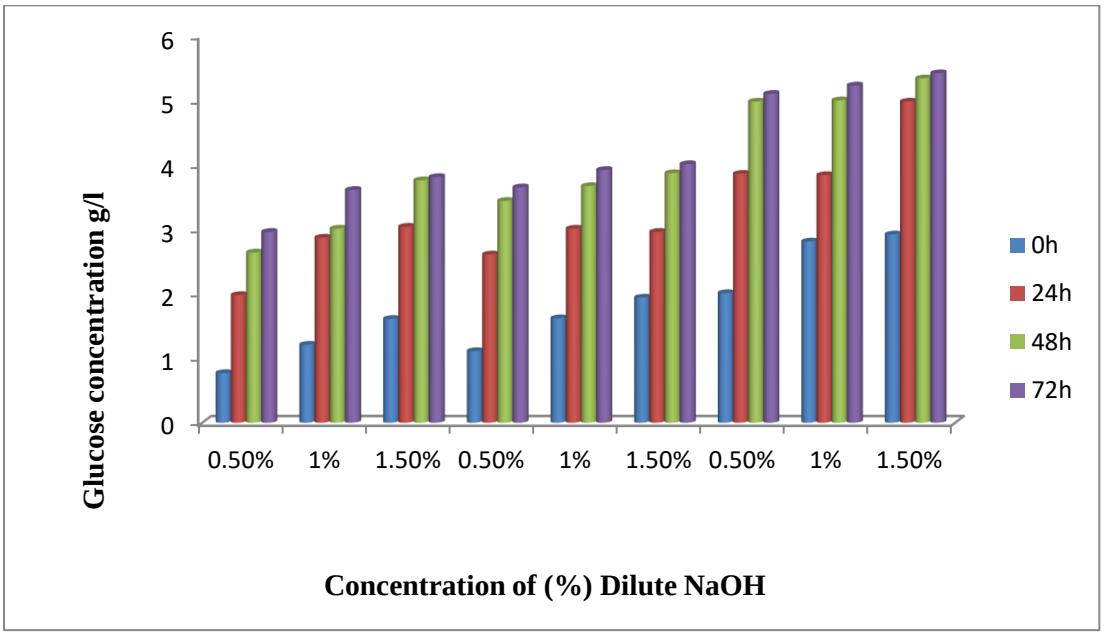

Figure 3. High glucose yield obtained from Wheat straw byNaOH pretreatment conditions at 120 ^{0}C

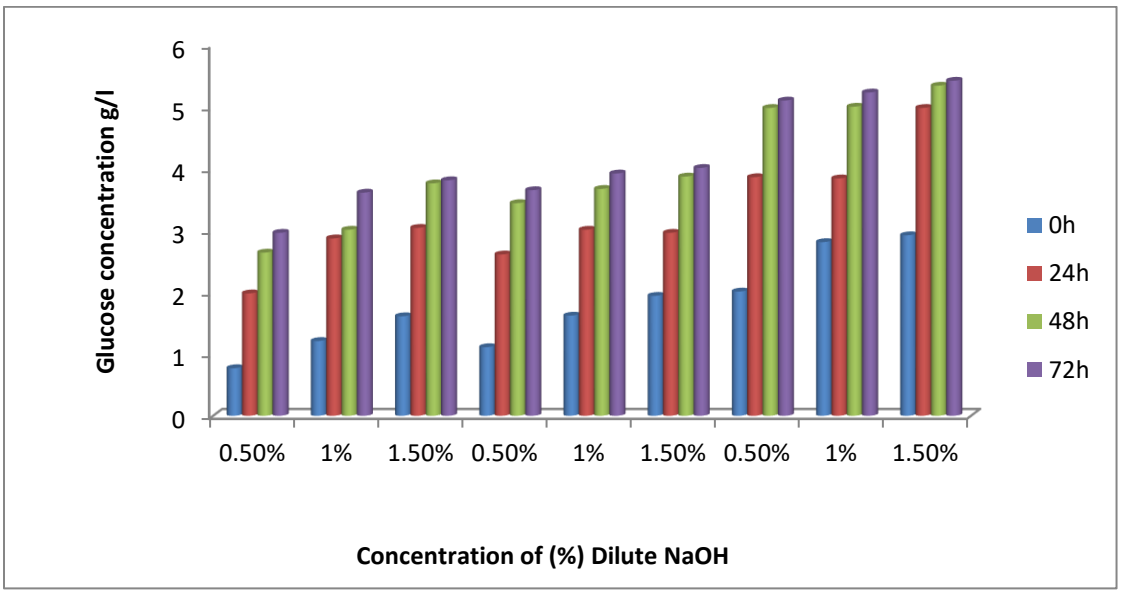

Figure 4. Glucose yield obtained from Rice straw at NaOH pretreatment conditions at 100 ^{0}C

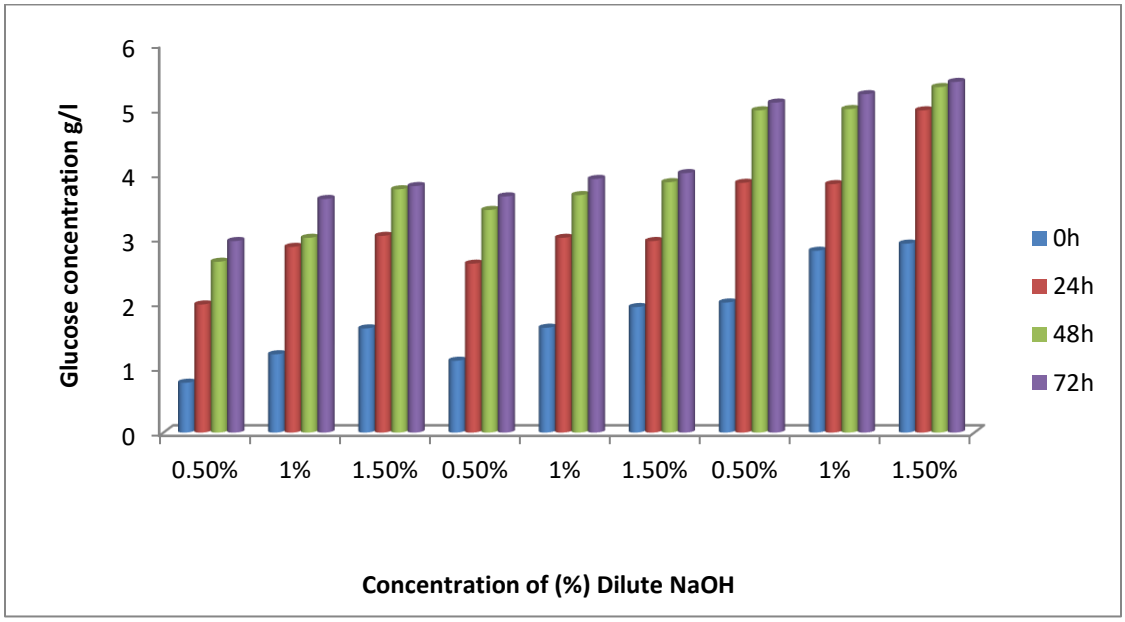

Figure 5. Glucose yield obtained from Corn stover byNaOH pretreatment conditions at 100 ^{0}C

HPLC Analysis of Enzymatic Hydrolysate

The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stoverwere further analyzed by HPLC. For this purpose, the samples those have shown higher amount of glucose at optimized conditions were used for analysis. Thesamples those were withdrawn at different time periods during enzymatic hydrolysis, then these were centrifuged at 14,000 rpm, at 4 ^{0}C for 15 minutes.Supernatant was separated and then filtered by using 0.22 µm syringe filter. An aliquot of the sample (500 µl) was diluted with 1ml methanol to bring the concentrations of the samples within the range of calibration curve. Methanol was used due to the solubility of the sugars. All the samples and standard solution of glucose was passed through the 0.22 µm filter prior to analysis. About 20µl of agrowaste sample was injected through injection loop into HPLC system. In order to analyze the glucose, enzymatically hydrolyzed samples were run in the gradient mode for 10 minutes (Shields and Cathcart.2010).

The identification of peak as based on the retention time t_R.Identification of glucose in three samples i.e. wheat straw, rice straw and corn stover were confirmed by the known standard injected through HPLC and its only one prominent peak was observed at a retention time of 3.255 minutes (Table 13 and Figures 19-20).

Table 10; Analysis of wheat and rice straws samples for sugars with HPLC

Components	**Retention time (min)**	**Concentration (mg/ml) Rice straw**	**Concentration (mg/ml) Wheat straw**
Glucose	8.6	22.52	28.3
Cellobiose	7.1	1.02	1.05
Xylose	11.6	4.3	5.6
Arabinose	12.0	1.4	1.8
Mannose	13.2	1.5	2.8
Galactose	15.5	1.2	1.5
Furfural	42.5	1.4	2.65
HMF	28	1.2	2.84

Analysis of sugar with HPLC

Fermentation with*Clostridium acetobutylicum*

The major product of this type of fermentation is known as ABE (acetone, butanol and ethanol) fermentation. The ratio of the acetone, butanol and ethanol in the fermentation process ismostly 3:6:1 as reported earlier by many authors. It was estimated that *Clostridium acetobutylicum* yields higherbutanol quantity at acidic pretreatment conditions as compared to alkaline pretreatment conditions. Although alkaline pretreatment conditions are best for butanol production because the chances for the production of fermentation inhibitors are very low. But in this experiment the reason for low butanol production might be due to low quantity of glucose obtained at alkaline conditions.Among three substrates the yield of butanol was high in wheat straw because of number of factors i.e. the amount of carbohydrate was high in wheat straw as compared to rice straw and corn stover. Low lignin content in wheat straw is responsible for high glucose yield as well as high yield of butanol than other two substrates. Wheat straw contains low lignin contents as compared to rice straw and corn stover. The higher butanol production from wheat straw may be due to its low lignin contents. Among three substrates wheat straw yields highest quantity of butanol at acidic pretreatment conditions. It was estimated from previous studies that wheat straw hydrolysate contain furfural and hydoxymethyl furfural that supported the production of biobutanol by fermentation.. It is concluded that wheat straw is a

superior fermentation substrate probably fermentation stimulatory chemicals are present in wheat straw.

Table 11; Acetone, Butanol and Ethanol production (%) from various agrowaste by *Clostridium acetobutylicum*

Samples	Acetone%	Butanol%	Ethanol%
Wheat straw	1.5	6.3	1.8
Rice straw	1.2	6.2	1.3
Corn stover	1.0	5.2	1.1

ABE production from Biomass samples

Table 12; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at H_2SO_4 pretreated samples

Samples	Acetone %	Butanol%	Ethanol%
Wheat straw	2.5	7.2	2.1
Rice straw	1.7	4.9	2.2
Corn stover	1.1	4.5	2.3

ABE production from Biomass samples

Table 13; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at NaOH pretreated samples

Samples	Acetone %	Butanol%	Ethanol%
Wheat straw	1.9	4.9	2.8
Rice straw	1.5	4.6	2.4
Corn stover	1.3	4.2	2.2

ABE production from Biomass samples

DISCUSSION

For the production of alcoholic fuels (Butanol and Ethanol) from lignocellulosic feedstock required various technological steps like acid or alkali pretreatment, saccharification and fermentation. To accomplish an cost effective production of biofuels,proper adjusting of all units of system is of great important. In the past different countries significantly improved alcoholic fuels production by refining different process like pretreatment, enzymatic hydrolysis, fermentation, and higher level of ethanol recovery (Zhao, 2012). The popular cases of biomass based fuels production in developed countries may be good references for the developing countries . In addition many novel ideas, such as biorefinery and the concept of oriented conversion of classified composition have been investigated for ethanol production. Similar technology are also applicable for butanol production fromlignocellulosic biomass (García et al., 2011; Demirbas, 2009). The cost of fuels may further decreases when it will produce at industrial scale and efficient combination of these processes will result in competitive biofuel production from plant biomass, which is currently not being utilized effectively (Talo et al., 2014).

Fermentation of available sugars in cellulosic biomass have potential to provides important products like acetone, butanol, ethanol and similar other alcohols, that could be used as liquid fuels. Mostly available source of biomass containing carbohydrates are wood wastes , agriculture crops like wheat, rice and cotton straws , corn covers, sorghum straws, fruit and vegetable wastes and similar other substrates. Cellulose is considered as major sugar for alcohol (fuel) production and cellulose is complex sugar present in plants materials. This complexcellulosic material is break down into smaller units with help of acid treatment and

enzymatic hydrolysis as well as bacterial/ fungal fermentation. These forms of alcohols is important because that may use as fuels. Therefore biofuels may provide solution of (1) combating climate change, as it help to reduce level of carbon emission release from traffic etc. (2) . Biofuel is able to respond growing demand of fossil fuel and energy (3) Biofuels securing energy supply as it provides security to challenges rising for fuels globally (4).Reducing amount of waste and utilizing natural resources, therefore biofuels is excellent example to provide answer of circular economy . In current study various cellulosic materials was used to produce bioethanol and biobutanol. Therefore various order of alcoholic fuels production from cellulosic substrates was obtained . Among all substrates of biomass used straws has provided better yields of alcoholic fuels as compared to others material used.However, amount of acetone, butanol and ethanol produced depends on nature of cellulosic biomass used as well as various distillation process conducted after fermentation for purification of these type of alcohols.

CONCLUSION

The country ‘s energy demand is expected to increase three fold by 2050, but supply position is not inspiring. Due to similar situation renewable and sustainable energy resources are the best alternative of conventional fuels and energy sources . Bioconversion of lignocellulosic biomass into alcoholic fuels (butanol and ethanol) provides a sustainable and economical pathway . While, a deep understanding of fundamentals of various pretreatment processes and development of more efficient and economical fermentation processes needs continuing efforts. Moreover, the development of cost-effective detoxification, more efficient microbial strains are required. The processof integration and optimization to reducing energy consumption as well as to increase yields replace currently available fossil fuels those are already in process of depletion. Therefore scientists all over the world are observing different cost effective methods for alternative sources of energy especially by using cellulosic biomass. It is expected that these types of research work could be an important phenomena for the development of country by using indigenous resources in future.

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CHEMICAL CHARACTERIZATION OF BIOMASS FOR PRODUCTION HIGH VALUE , ENVIRONMENTALLY FRIENDLY BIO FUEL

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ABSTRAT

Second generation cellulosic biofuels offers a solution to reduce carbon emissions of traffic as well as generation of energy for domestic and commercial uses. A study was conducted to develop a approach for the management of agriculture as well as other organic wastes utilization for production of alcoholic fuels like bioethanol and biobutanol. Therefore cellulosic materials like wheat , cotton and rice straws, corn stover , fruit wastes and cogon grass were used in this study Biological and chemical pretreatments were compared for each substrates. Efficiency of bacterial enzymes for saccharification of agricultural substrates was evaluated. It is concluded that these bacterial enzymes have the potential to hydrolyze not only pure substrates but can also degrade agricultural wastes. It is expected that outcome of this study will help to increase production of biofuels and to reduce burden of foreigner exchange that is currently being utilize to import fossil fuel from other countries.

Key words; Bioethanol, Biomass, Green house gases, Climatic changes

Introduction

Exploration of sources for alternate energy have been increasedbecause of increasing concerns about energy security and climate change. The transportation sector plays a significant role for emission of greenhouse gases due to uses of fossil fuels, However, replacement of oil derived fuels such as ethanol could reduce environmental impacts and give advantages on social as well as economical levels (Humbird et al,2011). Various alternatives to generate sustainable biofuels are being investigated. Biological energy resources arelike bioelectricity, biogases, biodiesel and bioalcohols. Among these sources, bioalcohol shows a great potential to reduce the emission of greenhouse gases, decrease the dependence on fossil fuel and act as a chemical feedstock and fuel for transport (Dhamole et al., 2015).The production of bioethanol has been improved extremely because many countries are trying to reduce the import of oil, improving the quality of air and growing rural economics. The global ethanol production is 51,000 million liters(Renewable Fuels Association, 2007). Ethyl alcohol has some advantages as a fuel as it has higher oxygen contents. The higher oxygen level permits improved oxidation of hydrocarbons with successive reduction in aromatic compounds and carbon monoxide emission. Ethanol has greater octane rating properties (Thomas and Kwong, 2001).

Biomass is a vital energy resource in Pakistan because ofagricultural based country. The biomass produced in livestock and agriculture sector in the form of animal waste and crop remaining as sugarcane bagasse and rice husk (Amiri et al. 2014; Chaudhry *et al*., 2009). Second generation biomass is mainly composed of lignocellulosic material. Lignocellulosic biomass is more plentiful organic substance on earth and consists of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%)(Huber et al., 2006).Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds (Demirbas, 2001). Most commonly used feedstocks are wheat straw, wheat bran, corn stover, corn steep liquor and apple pomace (Ejezi*et al*., 2006).Now a day, agricultural waste is used for the production of biofuels like biodiesel, bioethanol, biohydrogen and methane as compared to energy crops because they have competition with food crops. As huge amount of agrowaste is available and have discarding problem so, alternate option is the utilization of lignocellulosic biomass in order to reduce the competition between fuel and food (Mahro and Timm, 2007). The grasses are considered as reliable substance for extraction of ethanol. The utilization of perennial grasses is advantageous and possibly it further decreases the cost for the production of ethanol and its use as fuel (Gomez et al*.,* 2008).

The Cogon grass (*Imperatacylindrica*) can be grown all over the yearworldwide, particularly in subtropical as well as tropical countries. Cogon grass has been exploited to rise the soil stability and as fodder, it is recognized as worst weed and it is known as pest by almost 73 countries in all over 35 crops. The roots of cogon grass have secondary metabolites which have medical importance. It is known as perennial grass and could be cultivated in any soil which usually considered as unfit for production of crops. The cogon grass could be utilized as a raw material for renewable source of energy (Lin and Lee, 2011).The *Cynodondactylon* (Coastal Bermuda grass) is perennial grass that has the higher cellulosic content and can also be used for ethanol production. The excellent raw material for yield of ethanol is coastal Bermuda grass as it is either sold at a very cheap price or is wasted in most of the cases. Comparison of corn and Bermuda grass has shown that most potential source for production of ethanol is Bermuda grass because of higher contents of biomass and conversion of whole carbohydrates into bioethanol. Bermuda grass is predominantly present in tropical and subtropical part of the world. It reaches 1-30 cm in height and have deep roots up to 2m into the ground, however various roots penetrated less than 60 cm under-ground (Sun and Cheng, 2005).Even though some species of Bermuda grass can grow up to 15-20 cm, others may reach a height of greater than 1m long. Bermuda grass can naturally grow in many continents such as North Africa, southern Europe, Asia and Australia (Sluiteret al., 2008).

In the present study various agrowaste samples such as wheat, cotton and rice straws as well as corn stover, cogon grass and peels of fruit wastes were selected as these are main organic wastes in Pakistan. Other than wheat and rice the third vital cereal is corn. Its production is 4.695 million tons annually and grown in 1130 thousand hector. The second major crop is cotton and it is cultivated annually. The third important crop is rice and it is cultivated at 2891 thousand hector and it is produced 7005 thousand tons annually (PES, 2014-15). Various studies reported that 2.7 million tons waste such as rice straw, rice husk, canola straw, wheat straw, cotton stalks, cotton bagasse and sugarcane remains are cultivated in Sanghar which is known as agricultural area in Sindh region of Pakistan. Almost 75-85 percent of these feedstocks are not utilized are burnt away. So, these materials could be used for production of energy and having no effect on food or other domestic resources (UNEP, 2011).Maize (*Zea mays*) is the best of the cereal stovers and very abundant livestock feed. It can be grazed off otherwise, it is mostly burned in fields in many areas of Pakistan before next crop to sow and all parts of maize are usable for different purposes (Kim and Dale, 2004). Termite gut has one of the highest microbe densities on earth. Termites depend on the microbes in their gut or digestive tract to digest the complex sugars in wood into simpler molecules . Cellulose is a major sugar in wood , it is broken down by bacteria available in gut of termite and finally converted into various products including fatty acids and alcohol like ethanol etc.(Kim and Haltzapple.2005).*Clostridium* **,**genus of rod shaped gram positive bacteria member of which found in soil, water and intestinal tract. *Clostridium acetobutylicum* ferments sugar to a mixture of organic solvents like acetone , butanol and ethanol.***Saccharomyces cerevisiae*** (known as baker s years) single celled eukaryotes which is frequently used in fermentation process for production ethanol and other alcoholic products.

MATERIAL AND METHODS

Collection of Agricultural Substrates

Various samples of wheat and rice straws as well as, peel wastes were collected from various areas . About 1 Kg samples of each samples were collected in fine plastic bags. The samples were shad followed by sun and oven dried for overnight at 55 °C. The samples were converted into fine powder form by electric grinder and passed through 40 mesh standard size

sieve. The powder form of samples were saved in fine plastic bags duly labeled with the name and were stored in refrigerator at 4°C till further uses.

Proximate Analysis of Samples

All samples were analyzed for ash contents, volatile matter, crude protein, crude fiber, crude fat and wet as well as dry weight (AOAC,1990). The standard methods were used for the estimation of total solids and moisture contents by drying at 105 ºC to remove moisture from the samples (Sluiter, 2005).

Chemical analysis of raw biomass

The cellulose content of sample was estimated by using reported method. The hemicellulose was determined by computing ADF (Acid Detergent Fiber) and NDF (Neutral Detergent Fiber) differences .The lignin contents were determined by standard method as reported by AOAC (1990).

Analytical procedures

The fermentation products like monomer sugars (hexoses and pentoses) acetone- butanol and ethanol as well as their bio products were determined by using reported method (Haifeng et al., 2015).

Chemical Pretreatment

For chemical pretreatment two chemicals were used such as acid (H_2SO_4) and alkali (NaOH). Pretreatment experiment was performed by using H_2SO_4 and NaOH (1.0, 1.5 and 2%) at diverse temperatures such as 100 °C , 110 °C and 120 °C for different times durations (15, 30, and 45 minutes). Solid sample (10 %) (w/v) in reagent bottle was utilized during experiment. After pretreatment, the vacuum filtration assembly was used for filtration of sample in each bottle and the contents were emptied on filter paper. After filtration, the solid washed away with 300 ml

distilled water in order to neutralize the pH. The filter paper was than dried at 105 °C and weighed.

Figure 1. Biomass

Enzymatic Hydrolysis

The biomass after pretreatment 5% (w/v) was hydrolyzed with cellulose and β-glucosidases at 50 °C and 160 rpm for 72 hours in a water bath shaker with 0.05 M buffer (sodium citrate) at 4.8 pH. Cellulases having activity of (30FPU g-1). The samples were withdrawn from reagent bottle after every 12 hours to determine the concentration of sugar. After enzymatic hydrolysis, H_2SO_4 (µl) was added. Un-hydrolyzed sample was separated by centrifuging for 10 minutes at 13,500g. Supernatant was collected by means of syringe filters for sugar analysis by dinitrosalicylic acid (DNS) method .The amount of sugar was analyzed by p-hydroxybenzoic acid hydrazide (PAHBAH) method. By using the concentration 1Mm-25mM of xylose the standard curve was

drawn. Then by comparing the standard sugar concentration, the amount of sugar in pretreated sample was determined. The best pretreatment condition was selected after enzymatic hydrolysis process. The sample containing higher amount of released sugar was further selected for fermentation process. The solid biomass was stored at 4 °C which was then used for fermentation process .

Saccharomyces cerevisiae strain was maintained on YPD (yeast extract 1% (w/v), peptone 2% (w/v) and glucose 2% (w/v)] agar medium at 4°C. Culturing of yeast cells was carried out in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30°C for 16 h on a rotary shaker (100 r.p.m.) according to (Alfenoro, 2002)

Separate Hydrolysis and Fermentation

Fermentation experiment was carried out by using *C. thermocellum*grown in glucose yeast extract broth medium for 48 hours and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature (Jiang et al., 2015).Fermentation experiment was performed at 50^0C and 120 rpm for 72 hours under anaerobic conditions. After completion of fermentation reaction, the obtained mixture contains methanol, butanol, ethanol and acetone were removed by fractional distillation process in a fractional distillation apparatus on the basis of boiling point. As butanol has higher boiling point (118 ^{0}C) than water (100 ^{0}C) Butanol can be condensed then separated. The boiling point of ethanol is lower (78.3 ^{0}C) in comparison with water that's why it can be condensed earlier than water (Kathleem et al., 2018).

).

Statistical analysis

Data generated through various analysis were statically analyzed for mean, standard deviation etc.,

RESULTS AND DISCUSSION

Results regarding isolation of bacteria , chemical analysis of biomass samples as well as fermentation of sugars into acetone- butanol- ethanol are given in the following sections.Termites are considered as good source of various useful bacteria isolates those have industrial applications . These isolates are found to have good potential for conversion of various sugars into alcoholic products . Therefore in current study acetone- butanol - ethanol (ABE) were produced from organic wastes material of agriculture and municipal sources by using termite based bacterial isolates (Figures 2-3).

Biological Pretreatment

Results displayed in table 3 indicates amount of sugar released by different bacteria isolates. It was observed that bacterial isolates 9x (xylanase enzyme)has provided higher amount of sugar (27.84$\pm$0.48mM/l) from wheat straw (Table 3), which was higher than all other substrates analyzed.

Table1.Chemical pretreatment of biomass samples with different concentrations (%) of NaOH and H_2SO_4,to release of sugars (%).

Substrates	Chemicals					
	H_2SO_4 concentration			NaOH concentration		
	1%	2%	3%	1%	2%	3%

Wheat straw	15.38±1.24	19.74±1.25	6.38±0.86	14.71 ±0.46	15.95±0.08	16.85±0.15

Bacterial	**Corn stover**	**Wheat straw**

Corn stover	14.57±0.18	13.73±1.12	4.27±0.81	13.69±0.46	13.65±0.08	13.82±0.14
Cotton stalk	1.53±0.04	1.35±0.05	0.28±0.02	0.86±0.06	0.87±0.11	0.97 ±0.01
Rice straw	16.85±0.15	15.38±0.17	3.44±0.15	15.07±0.17	14.32±0.28	13.39±0.59

Pretreatment of biomass samples

END PRODUCT ANALYSIS

Simultaneous Sccharification and Fermentation

It was observed that there are variation for growth of different isolates on differentsubstrates that might be due to availability of amount sugars and other similar byproducts

Table 2. Various fermentation products (mM/l) obtained from biomass samples

	Acetate	Formate	Lactate	Ethanol	Acetate	Formate	Lactate	Ethanol
Isolate 9x	1.15±0.06	_	1.41±0.18	5.73±0.28	3.04±0.65	_	1.65±0.79	3.34±0.41
Isolate 10	1.28±0.14	_	3.44±0.34	6.98±0.58	1.55±0.28	1.24±0.17	6.14±0.55	5.99±0.26
Isolate 31	1.29±0.34	1.98±0.39	8.57±0.59	9.21±0.54	1.72±0.07	1.63±0.28	3.58 ±0.26	6.43±0.49

Various Fermentation products

Biomass analysis

Data in table 6 represents various parameter found in biomass samples. Whereas ligno-cellulosic contents of the samples are given in table 7. It was observed that Cogon grass has higher cellulosic contents as compared to other substrates used for analysis.

Table 3; Proximate analysis (%) of biomass samples

Substrate	Dry Matter	Moisture	Crude Protein	Crude Ash	Crude Fiber

Peel(wastes)	92.41±0.48	7.53±0.34	7.93 ±0.23	5.91± 0.45	33.87±0.33
Cogon grass	93.11±0.27	6.89±0.26	5.12 ±0.21	9.18±0.34	35.41±0.42

Analysis of organic wastes samples

Table 4. Chemical analysis (%)of biomass samples

Substrate	**NDF**	**ADF**	**Hemicellulose**	**Cellulose**	**Lignin**
Peel(Fruit wastes)	79.6±0.51	52.1±0.31	26.3±0.34	29.6±0.67	21.5±0.43
Cogon grass	82.06±0.72	48.41±0.42	29.6±0.52	34.2±0.83	15.32±0.25

Mean ± standard deviation (n=3) NDF = Neutral Detergent Fiber and ADF=Acid Detergent Fiber

Table 5; Recovery of solid mass (%) due to treatment with Acid under various conditions.

Pretreatment conditions.		**Total solid recovery (g/100g dry biomass)**	
Time (min.)	**H_2SO_4Concentration**	**Peel (wastes)**	**Cogon grass**
15	1.0	63.52±0.20	76.86±0.61
	1.5	62.02±0.13	74.24±0.43
	2.0	57.11±0.34	70.33±0.25
30	1.0	56.61±0.43	68.86±0.48
	1.5	56.07±0.22	67.10±0.35

	2.0	52.37±0.20	64.76±0.24
45	1.0	53.07±0.32	64.62±0.42
	1.5	52.11±0.51	64.02±0.36
	2.0	51.23±0.44	63.50±0.56

Dilute H_2SO_4 pretreatment

The samples of the various biomass were pretreated with dilute acid 1, 1.5 and 2% concentration, an autoclave at temperature of 105, 120 and 135ºC for the period of 15, 30 and 45 minutes. The temperature 120 °C is considered best for both the samples while the retention time of 15 minutes was suitable for peel wastes and 30 minutes for cogon grass at the concentration of 1.5% and 1% respectively, these are the optimized conditions that was used for enzymatic experiment (Figures 6-7).

Fermentation

Cogon grass produces 10% of ethanol where as Peel wastes produces 7.4% of ethanol.as shown in Table 9. As the time period increases, glucose concentration was reduced but ethanol concentration was enhanced but up to certain time limit. However, after 72 hours glucose concentration was not sufficient to maintain the ethanol production. Higher cellulosic but lower lignin contents of cogon grass was compared to Peel wastes and it was found that these contents make cogon grass a better candidate for ethanol production.

Table 6. Ethanol production from Cogon grass and peel wastes samples

Sample	**Ethanol Production (% v/w)**
Cogon Grass	10.5
Peel wastes	7.4

Table 7; Sugars and other products (%) obtained from grasses

Substrate	Glucose	Xylose	Lignin	Dry matter	Moisture	Ash
Cogon grass	32.36. ±1.14	18.85 ±1.18	6.93 ±0.44	90.33 ±1.85	9.67 ±0.54	5.77 ±0.46
Peal wastes	27.32. ±2.15	15.37 ±1.13	4.75 ±0.54	92.46 ±1.24	8.56 ±0.55	4.89 ±0.58

% age values of various parameters of biomass samples.

Dilute sulfuric acid pretreatment of Peel wastes

Glucose content in peel waste was 27.33±2.15% (Table 10). There was increased in the glucose content after treatment (Figures 10 -11) The solid fraction of samples has given larger quantity of glucose when it was treated with dilute acid concentration (1.8 %) for 30 minutes and the temperature of reaction was maintained at 110 °C. It proved that moderate temperature and acid concentration play key role to enhance the glucose contents during pretreatment. Similar finding on acid hydrolysis of orange peel at low temperature has been reported by Talo et al. (2014).

Percentage decrease in xylose content in solid fraction of peel wastes after H_2SO_4Pretreatment

In peel wastes xylose content was 15.37%±1.13 (Table 10) and after pretreatment decrease in xylose content was observed. An acid concentration of 1.10% and incubation temperature of 110 °C for 30 minutes was found optimum to achieve minimum xylose in the solid fraction (Figure 11).With dilute acid pretreatment 100% removal of hemicellulose is

possible (Sun and Cheng, 2005). Wyman et al. (2005) has also reported that maximum xylose solublization occurs at moderate temperature.

Percentage increase in lignin content in solid fraction after H_2SO_4pretreatment

Contour plot (Figure 10 and 11) also indicates that optimum acid dose of 2.0% for 37 minutes at 125 °C is enough to get minimum lignin in solid fraction. Although the reaction has shown apparently 7.32% increases in lignin content but values in real sense was in decreasing order . The apparent increase in lignin content was due to huge removal of xylose after H_2SO_4 pretreatment. If the temperature is kept constant, then the solublization of lignin becomes higher with elevating reaction time to ascertain maximum value.

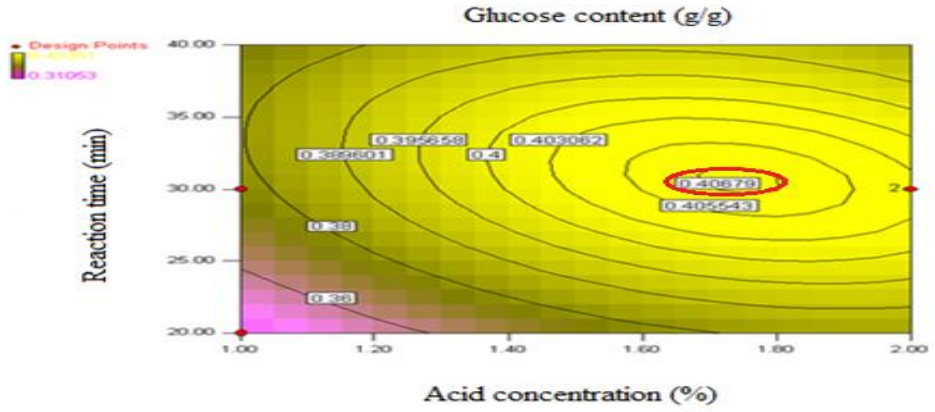

Figure 10; Contour plot for glucose content in relation to acid concentration and reaction time

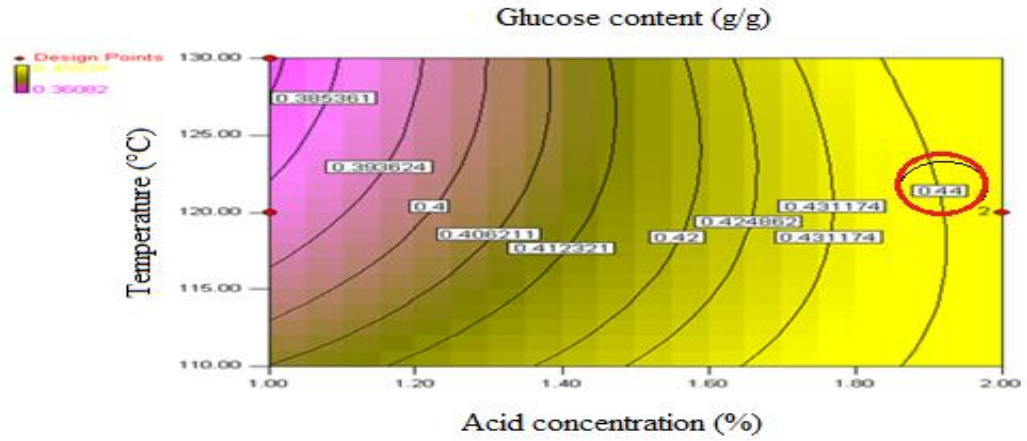

Figure 11; Contour plot for glucose content in relation to acid concentration and temerature

Table 8; Maximum Sugar yields after enzymatic hydrolysis of substrates at pH 4.8, 50 °C, 120 rpm.

Substrate	$Glucose_{SF}$ (2.5g/50mL)	$Glucose_{SF}$ (50g/L)	Glucose(g/L)	$Rate_{sac}$ (%)	$Time_{OPT}$(hours)
Cogon grass	1.10	22.00	17.72	80.54	72
Peal wastes	0.98	18.5	14.7	78.35	72

* SF = Solid fraction Y = Yield Sac = saccharification Opt = optimum

The reason behind higher saccharification (80.54%) was achieved as there was no accumulation of sugar like cellobiose occurred although cellobiose was available in reaction mixture. Xue et al. (2012) has also pointed out that the performance of celllases was actually enhanced (due to absence of cellubioses), and the results in higher sugar recovery after enzymatic hydrolysis.

Table 9 .Chemical analysis of various crops samples

Parameters	Cotton stalks	Corn stove
Moisture contents	6.5	7.0
Volatile Matter	77.0	75.0
Fixed Carbon	9.5	19.5
Ash contents	8.7	6.0
Crude Fiber	31.0	32.0
Ether extract	1.8	2.5
Crude Protein	4.2	3.8
Cellulose	34.5	33.6
Hemicellulose	29.5	32.5

Lignin	14.8	18.5

Various parameters of bioma

Analysis of Sugar after Pretreatment and Enzymatic Hydrolysis

In this study three different substrates i.e. corn stover, wheat straw and rice straw were used for sugar production by enzymatic hydrolysis in 500 mL Erlenmyer flask at 50 °C for three days. For sugar production, pretreatment process of lignocellulose is necessary to break down lignin and it increases the accessibility of enzymes and microbes to carbohydrates(Figures 12-13). Two types of pretreatment methods have been applied on these substrates. In physical pretreatment these substrates were first groundto fine powder and passed through 80 mesh size sieve to reduce the size of particles. Then these substrates were subjected to chemical pretreatment. In order to interrupt the structure of lignocellulosic biomass, acidic and basic pretreatment (Chemical pretreatment) conditions were applied (Table 12).

Dilute Acid Pretreatment

Different concentrations of sulphuric acid H_2SO_4 were used for pretreatment of agricultural waste samples. The samples were pretreated at a solid loading of 20% (w/v) slurry and heated at a temperature of 100, 110 and 120 ^{0}C in an autoclave. The reaction was performed at a retention time of 10, 15 and 20 minutes and at three different concentrations of sulphuric acid i.e. 0.5, 1 and 1.5%. At each temperature, substrate was pretreated with three different concentrations of sulphuric acid. A sample was pretreatedtriplicate at same temperature and reaction time. Total 9 treatments of three samples were pretreated at a time in 100ml reagent bottles. Total 9 experiments were done ($9 \times 9 = 81$) so, 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for acidic pretreatment (Figure 12).

Dilute Alkali Pretreatment

Dilute alkali was used for pretreatment of agrowaste samples. Different concentrations of sodium hydroxide (NaOH) were used at different temperature and different retention time to optimize the condition which may give maximum yield of glucose. The samples were pretreated at a solid loading of 20% w/v slurry and heated at 100, 110 and 120 ^{0}C in autoclave for 10, 15 and 20 minutes of reaction time. For pretreating the sample ofdifferent concentrations of sodium hydroxide 0.5%, 1% and 1.5% were used. Total 9 experiments were done (9 × 9 = 81) , therefore total 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for basic pretreatment.

Spectrophotometric analysis and Comparison of sugar production in three agrowaste samples

Better glucose yields were obtained from wheat straw in all 9 experiments after 72 hours of enzymatic hydrolysis. It was observed during experiment that by increasing the concentration of H_2SO_4 from 0.5 to 1.5% the amount of sugar was also increased. In all experiments, higher yield of glucose was also recorded at a retention time of 20 minutes rather than 10 and 15 minutes. For acidic pretreatment conditions of wheat straw, the conditions were optimized at 120 ^{0}C, 20 minutes of retention time with 1.5% of sulphuric acid (Figure 14). At this concentration, glucose concentration was at peak. During alkali pretreatment conditions, the glucose yield was increased by increasing the temperature and higher yield was recorded at 120 ^{0}C. Meanwhile at similar temperature high yield of glucose was recorded when 1.5 % concentration of NaOH was used (Figure 16) . By increasing the time of enzymatic hydrolysis from 0 to 48 hours sugar yield was increased but when the time is increased further to 72 hours sugar concentration was chopped.The decrease in glucose concentration was probably due to production ofinhibitors by higher acid concentrations. Maximum reducing sugars (7.73 g/L) were obtained at 120^{0}C , when 1% NaOH concentration and reaction time of 15 minutes were used .

Rice straw has shown higher glucose yieldin acidic pretreatment conditions at 110 ^{0}C, acid concentration (1.5%) and retention time 10 minutes was used (Figure 15).The optimum condition used for rice straw analysis in case of alkaline pretreatment, temperature (100 ^{0}C),

sodium hydroxide concentration (0.5%) and retention time (20 minutes) . Higher yield was obtained after 72hours of enzymatic hydrolysis (Figure 17).

During acidic pretreatment high yield of glucose was obtained at a temperature(120^{0}C), H_2SO_4 concentration (1.5%)and reaction time of 15 minutes (Figures 13-14). The optimum condition for corn stover at alkaline pretreatment condition was temperature 100 ^{0}C, concentration of sodium hydroxide (1.5%) and retention time applied was 20 minutes (Figure 18).

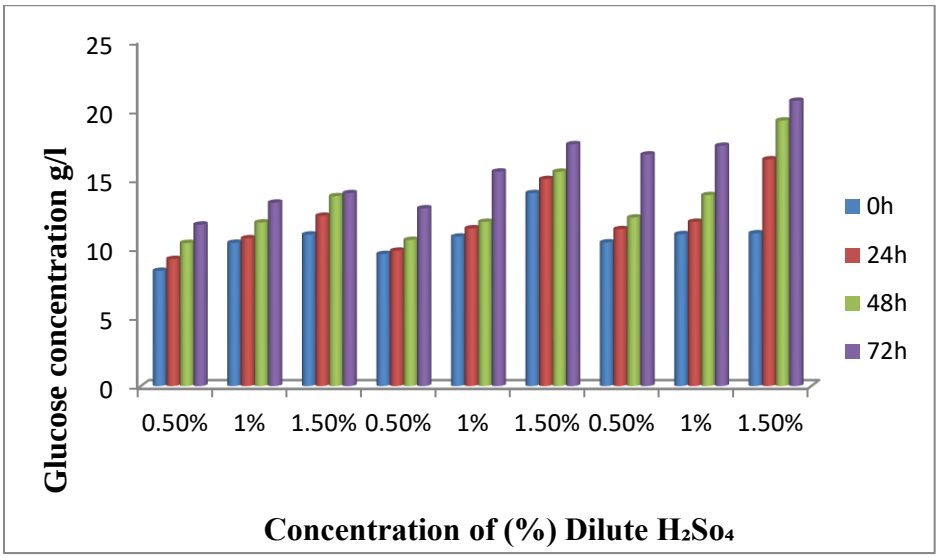

Figure 14. Glucose yield obtained from Wheat straw at H_2SO_4 pretreatment conditions at 120^{0}C

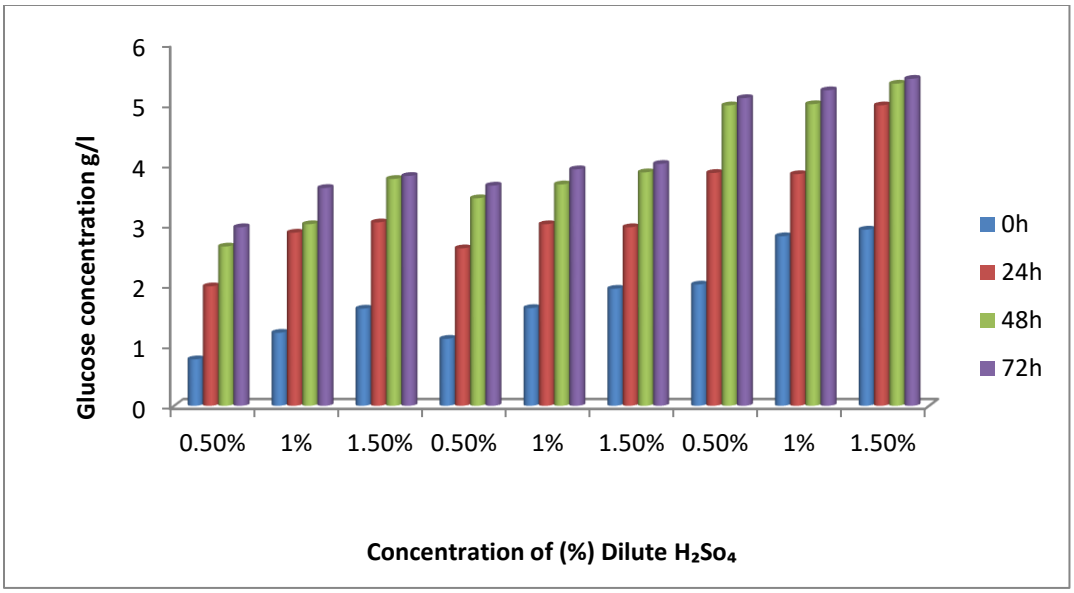

Figure 15. High glucose yield obtained from Rice straw at H_2SO_4 pretreatment conditions at 110 ^{0}C

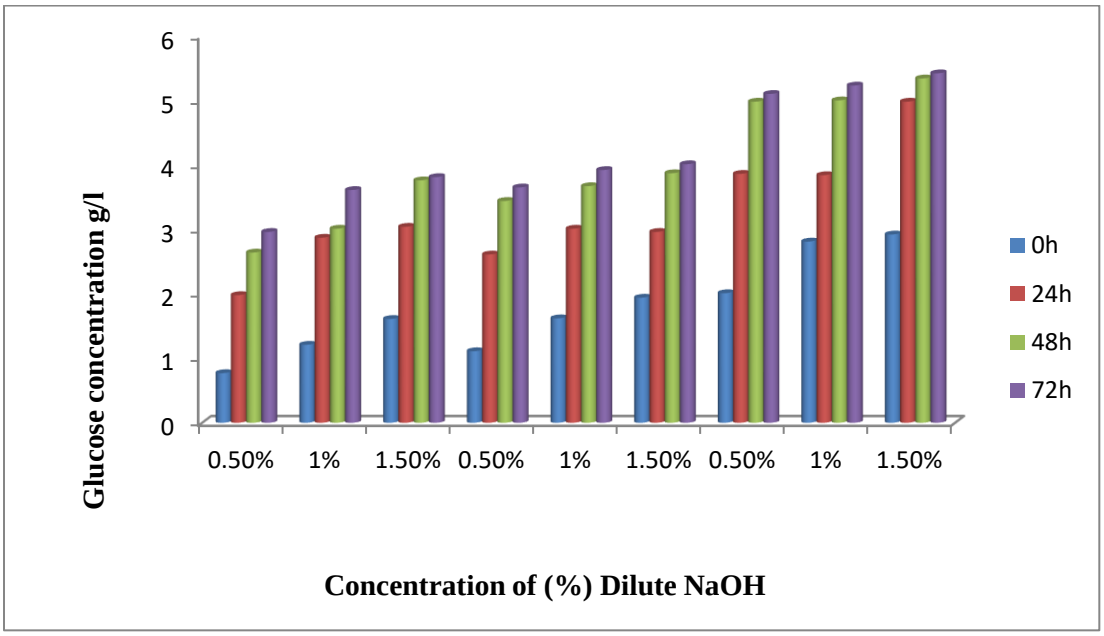

Figure 16. High glucose yield obtained from Wheat straw byNaOH pretreatment conditions at 120 ^{0}C

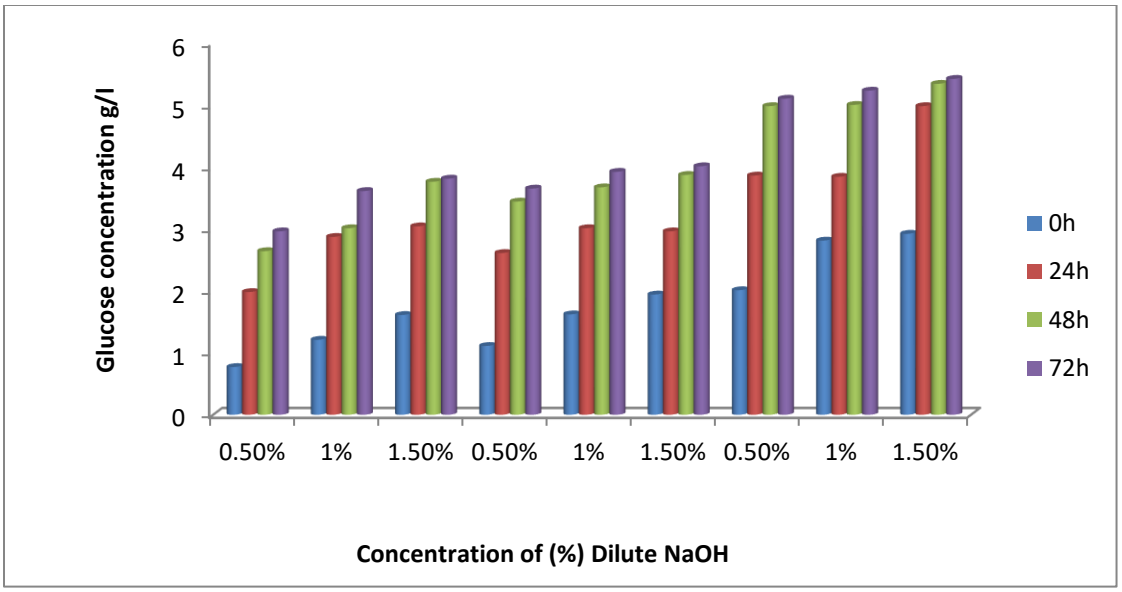

Figure 17. Glucose yield obtained from Rice straw at NaOH pretreatment conditions at 100 ^{0}C

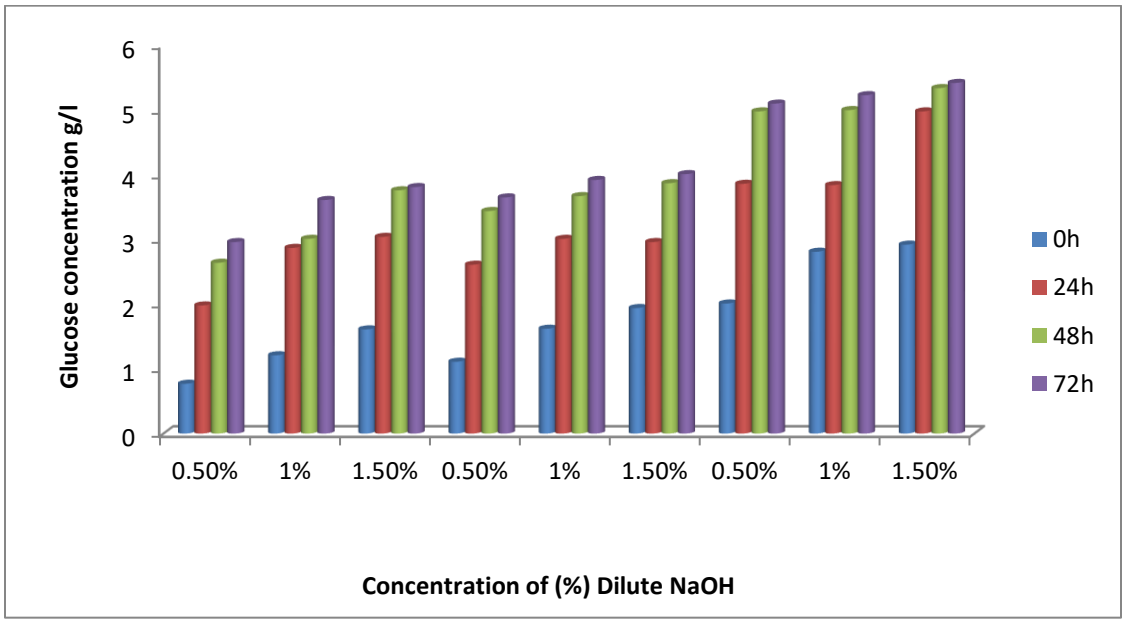

Figure 18. Glucose yield obtained from Corn stover byNaOH pretreatment conditions at 100 ^{0}C

HPLC Analysis of Enzymatic Hydrolysate

The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stoverwere further analyzed by HPLC. For this purpose, the samples those have shown higher amount of glucose at optimized conditions were used for analysis. Thesamples those were withdrawn at different time periods during enzymatic hydrolysis, then these were centrifuged at 14,000 rpm, at 4 ^{0}C for 15 minutes.Supernatant was separated and then filtered by using 0.22 µm syringe filter. An aliquot of the sample (500 µl) was diluted with 1ml methanol to bring the concentrations of the samples within the range of calibration curve. Methanol was used due to the solubility of the sugars. All the samples and standard solution of glucose was passed through the 0.22 µm filter prior to analysis. About 20µl of agrowaste sample was injected through injection loop into HPLC system. In order to analyze the glucose, enzymatically hydrolyzed samples were run in the gradient mode for 10 minutes (Shields and Cathcart.2010).

The identification of peak as based on the retention time t_R.Identification of glucose in three samples i.e. wheat straw, rice straw and corn stover were confirmed by the known standard injected through HPLC and its only one prominent peak was observed at a retention time of 3.255 minutes (Table 13 and Figures 19-20).

Table 10; Analysis of wheat and rice straws samples for sugars with HPLC

Components	**Retention time (min)**	**Concentration (mg/ml) Rice straw**	**Concentration (mg/ml) Wheat straw**
Glucose	8.6	22.52	28.3
Cellobiose	7.1	1.02	1.05
Xylose	11.6	4.3	5.6
Arabinose	12.0	1.4	1.8
Mannose	13.2	1.5	2.8
Galactose	15.5	1.2	1.5

Furfural	42.5	1.4	2.65
HMF	28	1.2	2.84

Analysis of sugar with HPLC

Fermentation with*Clostridium acetobutylicum*

The major product of this type of fermentation is known as ABE (acetone, butanol and ethanol) fermentation. The ratio of the acetone, butanol and ethanol in the fermentation process ismostly 3:6:1 as reported earlier by many authors. It was estimated that *Clostridium acetobutylicum* yields higherbutanol quantity at acidic pretreatment conditions as compared to alkaline pretreatment conditions. Although alkaline pretreatment conditions are best for butanol production because the chances for the production of fermentation inhibitors are very low. But in this experiment the reason for low butanol production might be due to low quantity of glucose obtained at alkaline conditions.Among three substrates the yield of butanol was high in wheat straw because of number of factors i.e. the amount of carbohydrate was high in wheat straw as compared to rice straw and corn stover. Low lignin content in wheat straw is responsible for high glucose yield as well as high yield of butanol than other two substrates. Wheat straw contains low lignin contents as compared to rice straw and corn stover. The higher butanol production from wheat straw may be due to its low lignin contents. Among three substrates wheat straw yields highest quantity of butanol at acidic pretreatment conditions. It was estimated from previous studies that wheat straw hydrolysate contain furfural and hydoxymethyl furfural that supported the production of biobutanol by fermentation.. It is concluded that wheat straw is a superior fermentation substrate probably fermentation stimulatory chemicals are present in wheat straw.

Table 11; Proximate analysis of straws samples

Substrates	**Dry matter%**	**Moisture%**	**Crude protein%**	**Crude fat%**	**Crude fiber%**	**Ash%**
Corn stover	91	5.32	7	2.9	2.5	3
Wheat straw	92.8	7.2	17.5	3.6	15	23.5
Rice straw	90.8	5.40	4.37	1.9	11	24

Analysis of biomass samples

Table 12;Chemical analysis of straws samples

Samples	Cellulose %	Hemicellulose%	Lignin%
Corn stover	30	21	7
Wheat straw	40	25	13
Rice straw	35	22	20

Chemical analysis of biomass samples

***Clostridium acetobutylicum*function for butanol**

The clostridium specie *Clostridium acetobutylicum*was maintained at at -20^{0}C. Enzymatically hydrolysed sample was then used for fermentation. The pH was maintained at 6.5 with NaOH. 1ml *C. acetobutylicum*spores were added in 100ml enzymatically hydrolysed solution in reaction bottle for separate hydrolysis and fermentation. These reaction bottles were placed in shaking incubator having 120rpm at 37^{0}C for 72 hours. The butanol concentration was determined by alcohol meter after 72 hours, and values were expressed as % butanol obtained from wheat straw, from rice straw and from corn stover. Among these three substrates wheat straw produced highyield of butanol (Tables 16 -18).

However, main end products of acidic /alkaline pretreatments and enzymatic hydrolysis are glucose and xylose which can be metabolized by clostridium species during growth and acetone – butanol- ethanol (ABE) fermentation (Qureshi and Blaschek, 2000; Moretti and Thorson, 2008).*C. acetobutylicum* and *C. beijerinckii* are two major microbes normally used in ABE fermentation. However, in recent years research worker from different countries have investigated various strain (s) their parent micorbes, cultivation conditions and growth media for various biomass samples used in batch, fed batch and continuous fermenters to produce ABE (Tables16-18)

Table 13; Acetone, Butanol and Ethanol production (%) from various agrowaste by *Clostridium acetobutylicum*

Samples	Acetone%	Butanol%	Ethanol%
Wheat straw	1.5	6.3	1.8
Rice straw	1.2	6.2	1.3
Corn stover	1.0	5.2	1.1

ABE production from Biomass samples

Table 14; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at H_2SO_4 pretreated samples

Samples	Acetone %	Butanol%	Ethanol%
Wheat straw	2.5	7.2	2.1
Rice straw	1.7	4.9	2.2
Corn stover	1.1	4.5	2.3

ABE production from Biomass samples

Table 15; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at NaOH pretreated samples

Samples	Acetone %	Butanol%	Ethanol%
Wheat straw	1.9	4.9	2.8

Rice straw	1.5	4.6	2.4
Corn stover	1.3	4.2	2.2

ABE production from Biomass samples

DISCUSSION

For the production of alcoholic fuels (Butanol and Ethanol) from lignocellulosic feedstock required various technological steps like acid or alkali pretreatment, saccharification and fermentation. To accomplish an cost effective production of biofuels,proper adjusting of all units of system is of great important. In the past different countries significantly improved alcoholic fuels production by refining different process like pretreatment, enzymatic hydrolysis, fermentation, and higher level of ethanol recovery (Zhao, 2012). The popular cases of biomass based fuels production in developed countries may be good references for the developing countries . In addition many novel ideas, such as biorefinery and the concept of oriented conversion of classified composition have been investigated for ethanol production. Similar technology are also applicable for butanol production fromlignocellulosic biomass (García et al., 2011; Demirbas, 2009). The cost of fuels may further decreases when it will produce at industrial scale and efficient combination of these processes will result in competitive biofuel production from plant biomass, which is currently not being utilized effectively (Talo et al., 2014).

Fermentation of available sugars in cellulosic biomass have potential to provides important products like acetone, butanol, ethanol and similar other alcohols, that could be used as liquid fuels. Mostly available source of biomass containing carbohydrates are wood wastes , agriculture crops like wheat, rice and cotton straws , corn covers, sorghum straws, fruit and vegetable wastes and similar other substrates. Cellulose is considered as major sugar for alcohol (fuel) production and cellulose is complex sugar present in plants materials. This complexcellulosic material is break down into smaller units with help of acid treatment and enzymatic hydrolysis as well as bacterial/ fungal fermentation. These forms of alcohols is important because that may use as fuels. Therefore biofuels may provide solution of (1) combating climate change, as it help to reduce level of carbon emission release from traffic etc.

(2) . Biofuel is able to respond growing demand of fossil fuel and energy (3) Biofuels securing energy supply as it provides security to challenges rising for fuels globally (4).Reducing amount of waste and utilizing natural resources, therefore biofuels is excellent example to provide answer of circular economy . In current study various cellulosic materials was used to produce bioethanol and biobutanol. Therefore various order of alcoholic fuels production from cellulosic substrates was obtained . Among all substrates of biomass used straws has provided better yields of alcoholic fuels as compared to others material used.However, amount of acetone, butanol and ethanol produced depends on nature of cellulosic biomass used as well as various distillation process conducted after fermentation for purification of these type of alcohols.

CONCLUSION

The country ‘s energy demand is expected to increase three fold by 2050, but supply position is not inspiring. Due to similar situation renewable and sustainable energy resources are the best alternative of conventional fuels and energy sources . Bioconversion of lignocellulosic biomass into alcoholic fuels (butanol and ethanol) provides a sustainable and economical pathway . While, a deep understanding of fundamentals of various pretreatment processes and development of more efficient and economical fermentation processes needs continuing efforts. Moreover, the development of cost-effective detoxification, more efficient microbial strains are required. The processof integration and optimization to reducing energy consumption as well as to increase yields replace currently available fossil fuels those are already in process of depletion. Therefore scientists all over the world are observing different cost effective methods for alternative sources of energy especially by using cellulosic biomass. It is expected that these types of research work could be an important phenomena for the development of country by using indigenous resources in future.

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CHERMICAL ANALYSIS OF SWERTIA CHIRAYITA AND THE IN *VIVO* ASSESSMENT OF ITS BIOACTIVITY

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ABSTRACT*:*

Swertia chirayita is medicinal plant which is widely used by Ayurvedic physicians because it contain many natural ingredients which are useful to cure many diseases.A study conducted to quantify the phytochemical from the plant. The Antioxidant activities of various extracts assessed by using DPPH assay while antimicrobial activities evaluated against bacterial strains by comparing with standard antibiotics.Finally on the basis of phytochemicals available in plants its bioactivity correlated further for its scientific use and development of lead compounds those required for human ailments.

Keywords : PhytochemicalsAntioxidant, Antimicrobial, Medicinal plants

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INTRODUCTION

The basic needs of primary health care system depends on availability of different drugs in the market. Traditional medicine is still the most manageable and affordable source of treatment. Different diseases treated by the medicinal plants in better way, either in the form of pure active principles or natural ways and sporadically used curative tonic in the developing countries. In the family Gentianaceae Swertia, a genus which comprise a huge group of annual and perennial herbs, expressing about 135 species. Swertia species have common ingredients for number of herbal remedies (Abbasi et al., 2915; Adedapo et al., 2009).Recorded 40 species of swertia in india, out of which chirayita is the most beneficial for its therapeutic actions. In 1993 Edward showed that prevalent uses of chirayita in traditional medicine give rise to over-exploitation from the natural environment. Ethnomedicinal studies identified swertia most bitter herb in its taste due to the existence of different chemical ingredients (Newman and Crag, 2012).

The swertia chirayita used as a drug constitutes whole plant in dried form collected in its flowering stage. The chirayita is known as a trade name and also the local name of the plant.

Swertia is well known for swiftly lowering fevers from colds and flues particularly malarial fever (Adedapo et al., 2009). Swertia is a powerful anti-inflammatory agent making it good for joint disease, swelling, pain, and redness. Swertia is excellent remedy for rheumatoid arthritis. Swertia also works well for treating liver cihrosis and also does a good job of detoxing the liver. Swertia also increases metabolism thus helping with weight loss(Beauchamp and Fridovich,1971)

. Swertia has good antidiabetic ,antianemic properties.Swertia species have xanthones as secondary metabolites which are used effectively to treat malarial fever and tuberculosis (Cefafelli et al., 2006; Demirtas et al., 2009). Scientists study antimicrobial activity of plant extracts when they agreed that conventional medicine can act as a substitute of healthiness as well as used as antibiotic (Dillard and German,2000) . Therefore keeping in view the importance of this herb current study designed to evaluate the phytochemical evaluation of swertia chirayita and invivo assessment of its bioactivity (Dufour et al.,2000; Flohe and Gunzler,1984).

MATERIALS AND METHODS

Collection and preparation of plant samples

Samples of swertia chirayita were collected from hilly area of Murree, in fine plastic bags duly labelled with data and time of collection of samples . After transportation of samples to laboratory , samples were properly identified by expert taxonomist and specimen was deposited for future reference (voucher no. 137). Samples were subjected to shad dried follow by sun and oven drying at lower temperature. Dried samples were ground to powder form and stored at lower temperature till further uses (Newman and Cragg,2012).

QUALITATIVE ANALYSIS OF PHYTONUTRIENTS:

Qualitative assessment of flavonoids, alkaloids, phenols, tannins, saponins glycosides, terpenoids and steroids was carried out in the precise ways (Harborne, 1998).

Test for alkaloids

Sample of 0.5 to 0.6 g finally mixed with 1%HCL of 8ml, filtered. Filtrate of 2 ml were taken and reacted with reagents (Abbassi et al., 2015), turbidity or precipitation showed the presence or absence of alkaloids.

Test for proteins.

a) Ninhydrin test: Crude extracts for protein test was heated with 2 ml of 0.2 % Ninhydrin solution and violet coloration specified the occurrence of proteins and amino acids.

Test for reducing sugars

a) Fehling's test: Fehling A and Fehling B reagents combine by equal parts and 2 ml from infusion taken further added to extract and heated till boiling. Reducing sugars identified by the appearance of reddish precipitate.

Test for carbohydrates

a) Benedict's test: Benedict's reagent of 2ml added in the sample and heated till boiling, magenta brownish precipitate seen showed the occurrence of the carbohydrates.

b) Molisch's test: Sample was made with 2.5 ml of Molisch's reagent and , 2 ml of concentrated H_2SO_4 was transferred to the test tube. violet ring will be formed at the junction of test tube indicating carbohydrate presence.

Test for Iodine

Iodine solution of 2ml added in the prepared sample. A dusky dark blue pigmentation showed the iodine indication.

Test for Saponin

Sample of 0.4-0.5 g was mixed in hot water in the test tube. Test tube will show mixture vigorously forming foam and the elevation of the foaming was noted to define the saponin amount in our sample. 3 drops of olive oil was added to the frothing and mixed strongly for the development of suspension , representative of saponins.

Test for flavonoids

a. Alkaline reagent test:

A solution of 2% of NaOH was added to Crude extracts of samples. Only few drops of acid give rise to yellowish colour mixture change into colorless form, directed the characteristic of flavonoids(Harborne, 1998).

ANTIOXIDANT ASSAY

DPPH radical scavenging activity assay

Each sample of 4mg prepared and its stock prepared in DMSO in different concentrations. 2.4mg of DPPH taken and made its volume upto 100 ml in methanol . Extract was tested first at single concentration and those showing good antioxidants activity were tested for different range of concentration to establish the EC50.100 µl of each dilution was taken in test tube along with 2ml of DPPH and one test tube was taken as blank .Negative control contain all reagent except extract. After 30 min absorbance measured at 517nm,experiment done in triplicate and ascorbic acid used as standard (Skehan et al., 1990 : Valko et al., 2006; Wu et al., 2006).

The ability of sample to scavenge DPPH radical calculated by following formula: DPPH Scavenging activity % = (A. control - B. sample) / (A. control)*100

ANTIMICROBIAL ACTIVITY:

Preparation of extract for antimicrobial activity

Preparation of samples extract for antimicrobial activity was defatted with petroleum ether and then consecutively prepared with water and methanol solvents with the help of soxhlet tool. The extracts we prepared then cooled at 25C,Puted for evaporation until sample completely dried and finaly got filtrate. The extracts we used for analysis dissolved in dimethylsulfoxide (DMSO) for antimicrobial assay with minor alterations (Upadhyay, 2015).

Test Organism

A panel of test organisms was used including Echerichia coli and gram positive Staphylococcus aureus,Pseudomonas aeruginosa, and streptococcus pyogenes were procured from National Institute of Health Islamabad.

Culture Media:

The antibacterial and antifungal studies were carried out using nutrient agar medium followed by refrigeration storage at 4^0C.bacterial strain grown at 37^0C maintained at nutrient agar slant at 4^0C.

EVALUATION OF ANTIBACTERIAL ACTIVITY:

The well diffusion assay technique (Upadhyay, 2015) used to evaluate the in vitro antimicrobial activity of all samples poured at 10µl of microbes culture added to petri plates.After media solidified hole was made by using 5mm cork and hole filled with20µl of plant extract(10mg/ml and 20mg/ml).The inoculated plate left for refrigeration and then put in incubator for incubation at 37^0C for 24 hrs .

RESULTS AND DISCUSSION:

Qualitative analysis of swertia chirayita showed the presence of alkaloids carbohydrates,flavonoids,glycosides,tanins/phenols,saponins and proteins but tanins found absent in chirayita sample 2 by ferric chloride and lead acetate test (Dillard and German, 2000).

Table.1. Qualitative analysis of selected parameters from plant samples:

Sr.No.	**Chemical constituents**	**Test Reagents**	**Chirayita 1**	**Chirayita 2**
1	Alkaloids	Dragendorff,s reagent	+ve	+ve
		Wagner,s reagent	+ve	+ve
		Mayer,s reagents	+ve	+ve
2	Carbohydrates	Molish Test	+ve	+ve
		Fehling Test	+ve	+ve
		Benedict Test	+ve	+ve
3	Flavonoids	Mg Ribbon and dil Hcl	+ve	+ve
4	Glycosides	NaOH Test	+ve	+ve
5	Tannins/Phenols	Ferric Chloride Test	+ve	-ve
		Lieberman,s Test	+ve	+ve
		Lead Acetate Test	+ve	-ve
6	Protein	Xanthoproteic Test	-ve	+ve
		Biuret Test	+ve	+ve
7	Starch	Iodine Test	+ve	+ve
8	Saponins	Frothing with Na HCO3	+ve	+ve

9	Amino acids	Ninhydrin solution	+ve	+ve

+Ve(positive), -Ve(negative

ANTIOXIDANT ASSAY:

Antioxidant assay confirms the in vitro antioxidant capacity of Methanolic extract is better compared with aqueous extract and had standard ascorbic acid *Chirayita* 2 has better scavenging capacity in methanolic extract as compared to aqueous extract.In aqueous extract chirayita 1 has better antioxidant activity (Schwartsmann et al.,2002; Valko et al., 2006).

Table 2. Antioxidant activity(DPPH assay) of aqueous extract of *Chirayita*:

Concentration (µg/ml)	**Ascorbic acid**	***Chirayita* 1(%inhibition)**	***Chirayita* 2(%inhibition)**
50	73.41±1.95	53.36±2.17	50.25±0.85
40	70.80±2.64	51.2±1.22	49.5±0.63
30	56.19±3.6	49.93±1.20	47.75±0.88
20	43.59±4.60	46.49±0.58	42.75±0.47
10	30.27±7.67	42.06±0.96	40.04±0.75
IC50	**25.83**	**35.30**	**44.89**

Table 3..Antioxidant activity(DPPH ASSAY) of methanolic extract of *Swerita chirayita* samples :

Concentration (µg/ml)	**Ascorbic acid**	***Chirayita* 1(%inhibition)**	***Chirayita* 2(%inhibition)**
50	71.9±0.74	75.38±0.89	65.15±0.13
40	68.18±1.74	69.19±5.89	59.79±0.50
30	55.79±3.28	52.80±3.33	48.15±0.80
20	46.75±1.29	37.7±1.35	42.54±0.51
10	35.04±5.005	21.36±13.20	36.52±0.66
IC50	**24.18**	**28.88**	**28.28**

ANTIMICROBIAL ACTIVITY:

Results showed that both samples have better antimicrobial activity with methanolic extract as compared to aqueous extract.

Table .4 Antibacterial activity of methanol extract of *Swertia chirayita*

Organism	**Extract/drug(µg/ml)/zone of inhibition(mm)**																	
	SCM 1				**SCM 2**							**Gentamycine**						
E.coli	-	-	-	-	10	20	-	-	-	10	20	25	18	20	23	26	28	30
S.aureus	-	-	-	-	-	1o	-	-	-	-	10	20	13	18	21	25	26	33
S.pyogns	-	-	-	-	10	20	-	-	-	-	-	10	19	21	23	27	31	32

p.aeuro-ginosa	-	-	-	-	-	20	-	-	-	-	10	20	-	-	1	3	8	14

SCM: *Swertia chirayita* methanol extract.: No activity.

Table 5. Antibacterial activity of Aqueous extract of *Swertia chirata*:

ORGANISM:	**Extract/drug(µg/ml)/zone of inhibition(mm)**																	
	SCA 1					**SCA 2**					**Gentamycin**							
E.coli	-	-	-	-	-	20	-	-	-	-	10	20	18	20	23	26	28	30
S.aureus	-	-	-	-	-	20	-	-	-	-	-	10	13	18	21	25	26	33
S.pyogenes	-	-	-	-	-	-	-	-	-	-	-	10	19	21	23	27	31	32

SCA: *Swertia Chirayita* aqueous extract. _ :No activity

CONCLUSION

Two samples of chirayita were under study for certain physiochemical parameters of its individual constituents. These parameters include quantitative aspects. Samples were collected from raining areas of Pakistan where people tradition being used since long history. The obtained results predicted the significant aspects of our objectives particularly the targets including antioxidants, antimicrobial properties of samples. All objectives showed significant range of results those are comparable to results reported by others on similar parameters.

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